VOLUME 31 OCTOBER 1953 NUMBER 5

Canadian Journal of Medical Sciences

Editor: J. B. COLLIP

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MEDICAL SCIENCES

(Formerly Section E, Canadian Journal of Research)

The CANADIAN JOURNAL OF MEDICAL SCIENCES is published bimonthly by the National Research Council of Canada under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. Matters of general policy are the responsibility of a joint Editorial Board consisting of members of the National Research Council of Canada and the Royal Society of Canada.

The National Research Council of Canada publishes also: *Canadian Journal of Botany, Canadian Journal of Chemistry, Canadian Journal of Physics, Canadian Journal of Technology, Canadian Journal of Zoology.*

EDITORIAL BOARD

Representing

NATIONAL RESEARCH COUNCIL	ROYAL SOCIETY OF CANADA	
DR. C. W. ARGUE (*Chairman*), Dean of Science and Professor of Biology, University of New Brunswick, Fredericton, N.B.	DR. G. M. VOLKOFF, Professor of Physics, University of British Columbia, Vancouver, B.C.	Section III
DR. G. E. HALL, President and Vice-Chancellor, University of Western Ontario, London, Ont.	DR. T. THORVALDSON, Dean Emeritus of Graduate Studies, University of Saskatchewan, Saskatoon, Sask.	
DR. T. THORVALDSON, Dean Emeritus of Graduate Studies, University of Saskatchewan, Saskatoon, Sask.	DR. D. L. BAILEY, Department of Botany, University of Toronto, Toronto, Ont	Section V
DR. W. H. WATSON, Professor and Head of the Department of Physics, University of Toronto, Toronto, Ont.	DR. E. HORNE CRAIGIE, Department of Zoology, University of Toronto, Toronto, Ont.	

THE CANADIAN ASSOCIATION OF PHYSICISTS	THE CHEMICAL INSTITUTE OF CANADA
DR. G. M. VOLKOFF, Professor of Physics, University of British Columbia, Vancouver, B.C.	DR. H. G. THODE, Department of Chemistry, McMaster University, Hamilton, Ont.

Ex officio

DR. LÉO MARION (*Editor-in-Chief*), Director, Division of Pure Chemistry, National Research Laboratories, Ottawa.	DR. H. H. SAUNDERSON, Director, Division of Information Services, National Research Council, Ottawa.

Manuscripts should be addressed to:

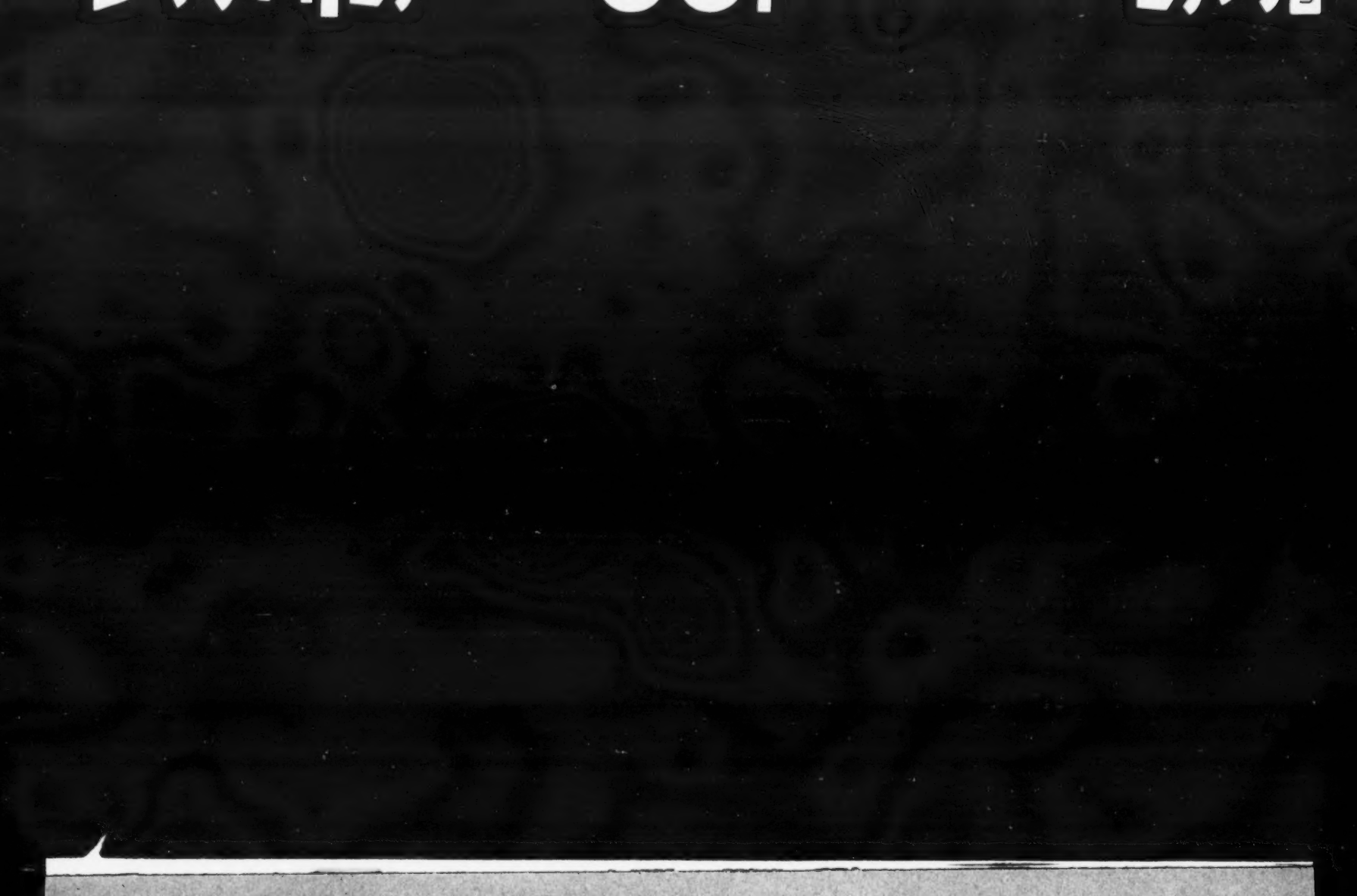
Dr. Léo Marion,
Editor-in-Chief,
Canadian Journal of Medical Sciences,
National Research Council,
Ottawa, Canada.

Each manuscript should be typewritten, double-spaced, and the original and one extra copy submitted (see **Notes to Contributors** inside of back cover).

Subscriptions, renewals, and orders for back numbers should be addressed to:

Administrative Services,
National Research Council,
Ottawa, Canada.

Subscription rate: $3.00 a year; single numbers: 50 cents. Special rates can be obtained for subscriptions to more than one of the Journals published by the National Research Council.



NOTICE

This is to announce that in 1954 the name of this Journal (Canadian Journal of Medical Sciences) will be changed to ***Canadian Journal of Biochemistry and Physiology.*** This change of name is made to indicate that the scope of the Journal has been broadened to include nonmedical biochemistry. The present sequence of volume numbers will be retained and publication will continue to be on a bimonthly basis. The first number to be published under the new name will be issued in January 1954.

The subscription rate for this Journal effective 1 January 1954 will be $3.00.

Canadian
Journal of Medical Sciences

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 31 OCTOBER 1953 NUMBER 5

SLIDE CULTURE OF TUBERCLE BACILLI

I. MEDIA AND METHODS[1]

BY ROGER W. REED[2]

Abstract

A modified slide culture technique is described for the cultivation of tubercle bacilli from untreated sputum specimens. Cultures are examined for growth under the low power after 6 and 14 days' incubation. Nearly 200 cultures of sputum from sanatorium patients were positive either on slide culture or on Herrold's egg-yolk agar. Sixty-seven per cent were positive by both methods. However, 87% were positive by slide culture while only 80% were positive on Herrold's. The greater sensitivity of the slide method was confirmed by quantitative studies. Slide cultures were positive in an average of 10 days, Herrold's agar cultures in 31 days. It is suggested that because of its technical simplicity and the rapid development of positive cultures, the slide culture method warrants further trial as a diagnostic procedure.

Introduction

The bacteriologic diagnosis of tuberculosis at present requires from three to eight weeks because of the relatively slow growth of the tubercle bacillus on conventional culture media. This delay is a severe handicap to those working in the field of tuberculosis, particularly in the clinical study of minimal disease. As a result of the mass roentgenographic surveys being conducted to discover tuberculosis in apparently healthy individuals, an increasing proportion of minimal disease is coming under observation (21). Accurate and early bacteriologic diagnosis is becoming increasingly important.

Microscopic examination of sputum is the most rapid and most economical method of demonstrating the presence of tubercle bacilli, but is only about 60% efficient in those with all stages of tuberculosis (22). However, in those with minimal tuberculosis, only 6% of smears were positive while culture or guinea pig inoculation showed the presence of tubercle bacilli in 69% of 269 cases reported by Decker *et al.* (7). Cultural studies are therefore necessary for bacteriologic diagnosis of minimal tuberculosis, for adequate observation of regression of the disease bacteriologically, and for periodic checking of arrested cases on out-patient status.

[1] *Manuscript received June 4, 1953.*

Contribution from the Department of Bacteriology, Dalhousie University, Halifax, and the Division of Laboratories, Department of Public Health, Nova Scotia.

[2] *Professor of Bacteriology, Dalhousie University and Provincial Bacteriologist.*

It is generally agreed that while present culture methods are fairly dependable, their slowness is a decided disadvantage. A number of media and methods have been devised in recent years in an attempt to induce more rapid growth of the tubercle bacillus. Dubos (8) and later Dubos and Middlebrook (9) described a new medium which produced rapid growth. Although this medium was not designed for primary isolation, its successful use for this purpose has been reported by Foley (10, 11) and by Goldie (12). One difficulty with the diagnostic use of Dubos's medium has been the very high rate of contamination of cultures which makes the validity of a negative culture questionable. This difficulty has been overcome to a large extent by Konowalchuk and Reed (16) who added 1 : 1,000,000 gentian violet to Dubos's medium prior to inoculation with sodium hydroxide treated specimens. Although growth was obtained in 7 to 10 days, there remained the disadvantages of treating the specimen before culture and of preparing and examining smears from each fluid culture for a final diagnosis.

A microculture method was described by Robert Koch in 1882 (14) by which he was able to demonstrate growth of tubercle bacilli microscopically at the end of one week. Modifications of this method were investigated by Pryce in 1941 (23) and Rosenberg in 1943 (26) but were too laborious and complicated for large scale diagnostic work. A number of reports on slide culture techniques for cultivation of tubercle bacilli have appeared in the European and South American literature (2, 3, 4, 17, 19, 20). Vaccaro and Alonso (27) and Koenigstein *et al.* (15) found the method equal or superior to conventional culture methods. Very little investigation of the slide culture technique has been carried out on this continent apart from the work of Berry and Lowry at the University of Colorado. Their reported results (5, 6, 18) indicate that slide culture methods may be useful in the diagnostic laboratory in providing a positive bacteriologic diagnosis of tuberculosis in six days or less. Their procedure is simple and permits the processing of large numbers of specimens by one or two technicians.

This paper describes a modification of the method for slide culture of *M. tuberculosis* reported by Berry and Lowry (5, 6, 18). However, some major modifications have been introduced which we have found to add to the efficiency of the method.

Methods

Choice of Medium

Kirchner's (13) medium was used as the basal medium. This is prepared as follows:

$Na_2HPO_4 . 12H_2O$	3 gm.
KH_2PO_4	4 gm.
$MgSO_4 . 7H_2O$	0.6 gm.
Sodium citrate	2.5 gm.
Asparagin	5 gm.
Glycerin	20 ml.
Distilled water	1000 ml.

Final pH is adjusted to 7.2. The medium is dispensed in 20-ml. amounts in special culture tubes and autoclaved for 20 min. at 15 lb. pressure.

Good growth of *M. tuberculosis* was obtained in Kirchner's medium enriched with 0.5% Bovine Albumin Fraction V (KA). However, this was considered too expensive an ingredient for a proposed diagnostic medium. Accordingly, 10% outdated whole bank blood (KB) and 10% fresh pooled human serum (KHS) were tried as alternative enrichments. At the same time a simple mixture of one part of bank blood to three parts of sterile distilled water (WB) was tried, as suggested by Oeding (19). Slide cultures for this series were prepared using 20 sputum specimens from a tuberculosis hospital. The results of this comparison are summarized in Table I.

TABLE I

EFFECT OF MEDIUM ON GROWTH OF *M. tuberculosis* IN SLIDE CULTURES AFTER 14 DAYS' INCUBATION

Code	Kirchner's medium plus:	No. of specimens	Technical failures	Amount of growth				
				4+	3+	2+	1+	Neg.
KA	0.5% Bovine albumin Fraction V	20	1	0	5	5	7	2
KB	10% citrated whole blood	20	2	9	5	2	0	2
KHS	10% fresh human serum	20	1	5	7	4	0	3
WB	1 part whole blood 3 parts sterile water	20	4	7	5	2	0	2

It is evident that growth of *M. tuberculosis* was most luxuriant in the blood-enriched media, including the simple blood and water mixture. However, the accumulation of red cells and cellular debris on the slides interfered with microscopic examination. KHS gave growth almost equivalent to that in the blood media, while poorest growth was obtained in KA. In further experiments, KHS gave much better growth than KA in 68% of 90 specimens cultured by the slide method. Dispersed growth in fluid media measured turbidimetrically also demonstrated the superiority of human serum over Fraction V albumin as an enrichment for Kirchner's medium. KHS was therefore chosen as our standard medium.

Since human serum is not available to all laboratories in the quantities that we were able to obtain from the Serology Department, fresh beef serum (KBS) and Difco dehydrated beef serum (KDS) were tested as supplements in comparison with human serum. It will be seen from Table II that KBS and KHS are equally good while KDS is of no practical use.

TABLE II

EFFECT OF HUMAN AND BEEF SERUM ON GROWTH OF *M. tuberculosis* AFTER 14 DAYS' INCUBATION

Code	Kirchner's medium plus:	No. of specimens	Technical failures	Amount of growth				
				4+	3+	2+	1+	Neg.
KHS	10% fresh human serum	20	0	12	4	0	0	4
KBS	10% fresh beef serum	20	0	13	3	0	0	4
KDS	10% dehydrated beef serum (Difco)	20	0	0	0	5	7	8

Procedure

It was essential to find a method of treating sputum which would not interfere with its fixation to the slide or with viability and subsequent growth of tubercle bacilli. Several variations of the Berry and Lowry method were tried and the following scheme has proved satisfactory:

(*a*) Sputum is homogenized in a laborarory model paint shaker. No digestion or concentration is necessary.

(*b*) Two thick smears of homogenized sputum are made on standard microslides with etched ends, and these are dried for one hour at 37° C. This treatment ensures adherence of smears to the slide during incubation and does not appear to affect growth.

(*c*) Slides are completely immersed in 6% sulphuric acid for seven minutes. This reduces contamination to a minimum (about 2%) without any apparent effect on growth of tubercle bacilli. Slides are then flushed three times with sterile distilled water to remove all traces of acid. (The decontamination tubes described by Lowry and Berry (18) are excellent for acid and water rinses.*)

(*d*) Slides are transferred aseptically to special culture tubes* (18) and incubated at 37° C. in the horizontal position with the smear up to provide maximum aeration.

(*e*) Following incubation, slides are removed from the tubes with forceps and fixed at 90° C. for 30 min. on a Cenco electric staining bath, stained, and examined.

(*f*) All specimens so cultured were also concentrated by the trisodium phosphate method and inoculated to Herrold's egg-yolk agar. These are referred to as 'routine' cultures and served as controls in all experiments.

Examination of Slides

Following fixation of slide cultures, a cold stain is applied according to the method of Aubert (1), using carbolfuchsin to which a few drops of "Tween 80"

* *Culture tubes and decontamination tubes were supplied by Fisher Scientific Company, Montreal, P.Q.*

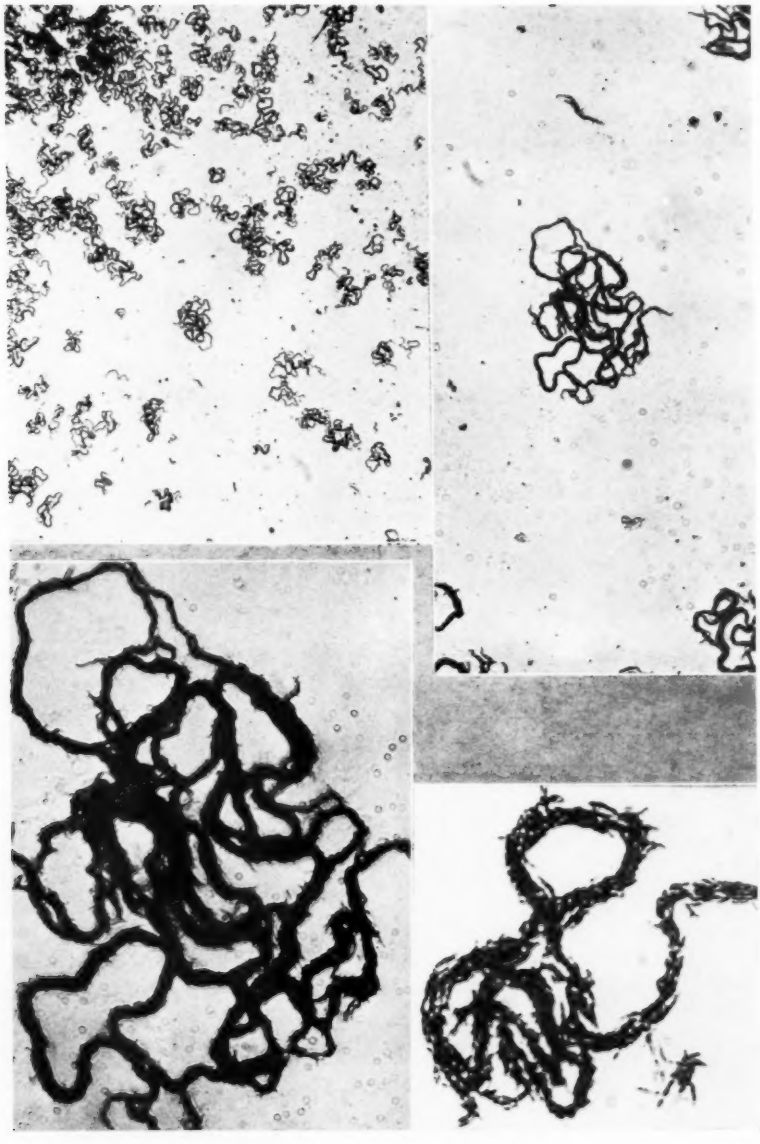


FIG. 1 (Upper left). Colonies of *M. tuberculosis* from a 14-day slide culture. (35×)
FIG. 2 (Upper right). A single colony from Fig. 1 enlarged to 120×.
FIG. 3 (Lower left). The same colony enlarged to 400×.
FIG. 4 (Lower right). A single colony from a six-day slide culture of *M. tuberculosis*. (1000×)

have been added. This stain is as effective as the Ziehl-Neelsen method, saves time, and avoids mess. Slides are examined under the low power (100×) for characteristic microcolonies of *M. tuberculosis*. The majority of freshly isolated cultures show the bacilli arranged in parallel in fasciculate, twisting configurations (Figs. 1–4) varying from 5 to 50 cells in width. Cultures from strongly positive specimens will often show growth visible to the naked eye as minute red dots on the slide, and occasionally growth is so heavy that the slide cannot be decolorized.

It is essential to examine the entire smear to be sure of a negative culture. This can be done in five minutes using wide-angle eye pieces. Under oil immersion, the typical morphology of *M. tuberculosis* is seen. Unless there is some doubt, however, it is rarely necessary to go beyond low power.

Treated vs. Untreated Sputum

A comparison was made of slide cultures prepared from homogenized but otherwise untreated specimens and those prepared using the sediment from digested concentrated sputum. Slide cultures were set up from 18 sputum specimens and growth assessed by the size and number of colonies per low power field. The results are shown in Table III.

TABLE III

COMPARISON OF SLIDE CULTURES PREPARED FROM TREATED AND UNTREATED SPUTUM

Amount of growth	No. of specimens	
	Untreated	Concentrated
4+	2	0
3+	6	2
2+	4	11
1+	0	2
Negative	6	3

More cultures from untreated sputum showed 4+ or 3+ growth than from concentrated sputum. Six of the former were negative, while three of the latter were negative, out of 18 specimens. There was a greater tendency for smears prepared from the concentrate to float off the slide during incubation. Because of this and the more luxuriant growth, it would appear that the additional time and manipulation involved in concentration procedures does not materially increase the effectiveness of the slide culture method.

Comparison of Slide and Routine Cultures

A total of 246 specimens of sputum obtained from patients in tuberculosis hospitals have been cultured by the two methods. Slide cultures were prepared from homogenized but otherwise untreated sputum. For the

preparation of routine cultures, sputum was digested overnight at 37° C. with an equal volume of 20% trisodium phosphate, neutralized, and centrifuged. The sediment was inoculated onto slopes of Herrold's egg-yolk agar. Slide cultures were read at 6 and 14 days; routine cultures were read weekly for 12 weeks. Smears of the concentrate were examined on all specimens. These results are shown in Table IV.

TABLE IV

COMPARISON OF SLIDE AND ROUTINE CULTURES OF SPUTUM

Conc. smear	Slide	Routine	Number	%
114 positive	+	+	91	80
	+	−	14	12.3
	−	+	5	4.4
	−	−	4	3.5
132 negative	+	+	37	28
	+	−	24	18
	−	+	20	15
	−	−	51	38

Of 114 specimens with a positive smear, 80% were positive by both slide and routine culture. It is of interest to note that while 4.4% were positive on routine culture only, 12.3% or nearly three times as many were positive by slide culture alone. In the group of 132 specimens negative on smear, 28% showed positive cultures by both methods while 3% more were positive by slide culture alone than by routine culture alone.

In Table V the entire series of 246 specimens is considered together, regardless of whether smears were positive or negative. Fifty per cent were positive by both methods, 15% were positive by slide only, and 10% by routine only. The total number positive by slide culture was 161 or 65.5% while 148 or 60% were positive by routine culture.

TABLE V

COMPARISON OF SLIDE AND ROUTINE CULTURES OF SPUTUM

Total spec. 246		No.	%
Total	Slide +, routine +	123	50
	Slide +, routine −	38	15.4
	Slide −, routine +	25	10.0
Total	Slide +	161	65.5
Total	Routine +	148	60

Of the 246 specimens, 191 gave positive cultures by one or both methods. The positive cultures alone are considered in Table VI. Of 110 positive cultures on specimens with positive smears, 83% were positive by both slide and routine methods. An additional 12.7% were positive on slide alone but

TABLE VI

COMPARISON OF RESULTS OF ALL POSITIVE SPUTUM CULTURES

Culture + by:	Smear pos. (110)	Smear neg. (81)	Total (191)
Slide and routine	91 (83%)	37 (45.7%)	128 (67%)
Slide only	14 (12.7%)	24 (29.5%)	38 (20%)
Routine only	5 (4.5%)	20 (24.7%)	25 (13%)
Slide (total)	105 (95.5%)	61 (75.3%)	166 (87%)
Routine (total)	96 (87.2%)	57 (70.5%)	153 (80%)

only 4·5% more were positive on routine alone. Eight per cent more positives were obtained by slide than by routine culture.

Eighty-one of the positive cultures were from specimens with a negative smear. Slide culture yielded 4.8% more positives than routine culture. When all positives are considered together, regardless of smear, it is seen that slide culture yielded 7% more positives than routine culture. Thirty-eight or 20% were positive on slide culture alone and only 25 or 13% on routine culture alone.

Time of Incubation

Earlier workers (5, 18) incubated slide cultures for a maximum of six days. We have used longer periods and find that two weeks' incubation yields twice as many positive cultures as does six days. Table VII shows the time required for positive cultures by the two methods on the same specimens. Of 162 positive slide cultures, 80 or 49% were positive in six days, but 100% were positive after two weeks' incubation. That is, 51% of the slide cultures would have been declared negative without the additional incubation time. Slides have also been examined at 8, 10, and 12 days but so few additional positives appeared that 6 and 14 days were chosen as the optimum times for readings. Only 6.25% of routine cultures were positive in two weeks.

TABLE VII

COMPARISON OF TIME REQUIRED FOR SLIDE AND ROUTINE CULTURES TO BECOME POSITIVE

No. of cultures	Incubation time in weeks								
	1	2	3	4	5	6	8	10	12
	Cumulative percentage positive								
Slide, 162	49	100	–	–	–	–	–	–	–
Routine, 144	0	6.25	40.3	73	80.5	89	94	97.8	100

The average time required for a positive slide culture in this series of 162 was 10.8 days compared with 30.6 days for 144 routine cultures. This represents an average saving of three weeks in reporting positive cultures.

Quantitative Comparison of the Two Methods

The results obtained on actual clinical specimens show that slide cultures yield more positives than do routine culture methods. It was felt that a quantitative determination of the minimum number of bacteria required to initiate growth by each method might lend further support to this finding.

A freshly isolated strain of *M. tuberculosis* was grown in fluid Dubos's medium for two weeks, then diluted to the density of MacFarland Nephelometer Tube No. 1 (300,000,000 per ml.). Viable counts were done by the surface drop-plate method of Reed and Reed (25), using the clear plating agar described by Yegian and Budd (28). Counts varying from 285,000,000 to 295,000,000 were obtained. Based on these counts, known numbers of tubercle bacilli from fluid Dubos's cultures were added to purulent sputum from bronchiectatic nontuberculous patients. This was homogenized and slide cultures were prepared. The sputum was then digested overnight at 37° C. with 20% trisodium phosphate, concentrated 50 times by centrifugation, and the sediment inoculated to the surface of Herrold's egg-yolk agar. Calibrated dropping pipettes (25) were used to add measured volumes to slides and to Herrold's medium.

The results of duplicate experiments done at different times showed complete agreement except in one case, as shown in Table VIII. Positive slide cultures were obtained on both occasions from an inoculum of 50 bacilli per slide while Herrold's agar cultures were negative to an inoculum of 62 bacilli. An inoculum of 125 bacilli per slope gave a positive culture in one experiment, a negative in the other. It is apparent that slide culture is more sensitive than

TABLE VIII

QUANTITATIVE COMPARISON OF SLIDE AND ROUTINE CULTURE METHODS USING KNOWN CONCENTRATIONS OF TUBERCLE BACILLI

Bacilli/ml. sputum	Bacilli/ slide	Slide culture		Bacilli/ slope	Routine (weeks)						
		6 days	14 days		2	3	4	6	8	10	12
1,000,000	50,000	+	+	125,000	−	+					
100,000	5000	+	+	12,500	−	+					
10,000	500	−	+	1250	−	+					
1000	50	−	+	125	−	−	+*	−	−	−	−*
500	25	−	−	62	−	−	−	−	−	−	−
100	5	−	−	30	−	−	−	−	−	−	−

* *Duplicate experiments.*

routine culture when the inoculum is in the neighborhood of 50 bacilli. Regardless of the size of inoculum, slide cultures become positive more rapidly than routine cultures. This work confirms the findings obtained on specimens of sputum from tuberculous patients.

Discussion

Recent reports of a rapid slide culture method for culturing *M. tuberculosis* from clinical specimens have led to a further evaluation of the method as a diagnostic procedure. The method described is simple, easily adaptable to routine laboratory use, and eliminates much of the manipulation required by conventional culture methods. A single technician can set up 50 specimens completely in three hours using this method. The preparation of media requires only a few minutes as compared to the time and care necessary for conventional media. Better growth is obtained when the basal synthetic medium is supplemented with 10% fresh human or beef serum than when 0.5% Fraction V Bovine Albumin is used. This makes a relatively inexpensive medium for large-scale diagnostic work.

Slide cultures appear positive in either one or two weeks, as compared with 3 to 12 weeks in the control cultures. It is generally agreed that in order to detect the maximum number of positive specimens, the use of more than one medium or method is to be desired. This view is supported in the present work by the appearance of a small proportion of cultures which were positive by routine methods but were negative by the slide method. However, where only a single culture method is feasible, fewer positives will be missed if only slide culture is used.

There are many circumstances under which this more rapid method of culture is both advantageous and humane. For example, predischarge sputum cultures of sanatorium patients can be done by the slide culture method and the results of several daily cultures known before the patient leaves hospital. Not infrequently, specimens taken on discharge show positive cultures by routine methods several weeks later. The patient must then be recalled for further treatment, a most disagreeable step for both patient and physician. In out-patients the disease can be followed more readily by the slide culture method and the patients called in for treatment much earlier, thus affording a measure of protection both to themselves and to their contacts. Minimal disease can be followed by daily slide cultures and the results known long before a single routine culture can be declared positive or negative.

Antibiotic sensitivity tests can be carried out rapidly at the same time as primary culture, as will be shown in a later paper (24). Currently some 100 unselected sputum specimens a month are being cultured by both slide and routine methods in the Tuberculosis Wing of Camp Hill Hospital (D.V.A.) in Halifax. The results of this comparison will be reported after a 12-month trial. In addition, the behavior of widely selected strains of known virulent, avirulent, and saprophytic acid-fast bacilli are being studied in slide culture. Other types of clinical specimens are also being cultured by this method.

Summary

A technically simple method of culturing *M. tuberculosis* from sputum has been described. Growth is obtained in 6 to 14 days on slides incubated in a serum-synthetic medium and is detected microscopically. The method appears to be more sensitive than conventional culture methods when used as a diagnostic procedure. The saving of up to three weeks in reporting positive cultures is worthy of note in special types of tuberculous patients. Further studies of the method are in progress.

Acknowledgments

The author is indebted to Mrs. Elizabeth Parsons for invaluable technical assistance. It is a pleasure to acknowledge the cooperation of Mr. D. M. Simpson, Bacteriologist, Camp Hill Hospital, Halifax; Dr. C. J. W. Beckwith, Superintendent, Halifax Tuberculosis Hospital; and Dr. J. E. Hiltz, Superintendent, Nova Scotia Sanatorium, Kentville, for supplying clinical specimens and for their interest and encouragement.

This investigation was supported financially by a Public Health Research Grant and in part by the Dalhousie Medical Research Fund.

References

1. AUBERT, E. Can. J. Pub. Health, 41 : 31. 1950.
2. BADOUX, J. Soc. vaudoise sci. naturelles, 64 : 277. 1950.
3. BADOUX, J. J. Med. Leysin, 29 (1) : 1164. 1951.
4. BERNARD, E. and KREIS, B. Rev. tuberc. (Paris), 13 : 124. 1949.
5. BERRY, J. W. and LOWRY, H. Am. Rev. Tuberc. 60 : 51. 1949.
6. BERRY, J. W. and LOWRY, H. Am. J. Clin. Path. 20 : 1037. 1950.
7. DECKER, W. P., ORDWAY, W. H., and MEDLAR, E. M. Am. Rev. Tuberc. 47 : 625. 1943.
8. DUBOS, R. J. Proc. Soc. Exptl. Biol. Med. 58 : 361. 1945.
9. DUBOS, R. J. and MIDDLEBROOK, G. Am. Rev. Tuberc. 56 : 334. 1946.
10. FOLEY, G. E. Proc. Soc. Exptl. Biol. Med. 62 : 298. 1946.
11. FOLEY, G. E. J. Lab. Clin. Med. 32:842. 1947.
12. GOLDIE, H. Proc. Soc. Exptl. Biol. Med. 65 : 210. 1947.
13. KIRCHNER, O. Zentr. Bakt. Parasitenk. Abt. I, Ref. 124 : 403. 1932.
14. KOCH, R. Berlin. klin. Wochschr. 19 : 221. 1882.
15. KOENIGSTEIN, R. P., CHENG, P. S., KOENIGSTEIN, K., and SUEN, I. D. Wien. klin. Wochschr. 63/38 : 723. 1951.
16. KONOWALCHUK, J. and REED, G. B. Can. J. Med. Sci. 29 : 263. 1951.
17. KRUML, J., PREROVSKY, I., TREFNY, Z., and ZUFFNICKOVA, J. Časopis Lékářů Českych, 89 : 841. 1950.
18. LOWRY, H. and BERRY, J. W. Am. J. Clin. Path. 20 : 273. 1950.
19. OEDING, P. Acta Tuberc. Scand. 25 : 208. 1951.
20. PARAF, J. and DESBORDES, J. Bull. mém. soc. med. hôp. Paris, 65 : 1355. 1949.
21. PARRAN, T. Am. Rev. Tuberc. 50 : 365. 1944.
22. PINNER, M. Pulmonary tuberculosis in the adult. Charles C. Thomas, Springfield. 1945.
23. PRYCE, D. M. J. Path. Bact. 53 : 327. 1941.
24. REED, R. W. To be published.
25. REED, R. W. and REED, G. B. Can. J. Research, E, 26 : 317. 1948.
26. ROSENBERG, K. S. Lancet, 1 : 615. 1943.
27. VACCARO, H. and ALONSO, G. Rev. Med. Chile, 79 : 31. 1951.
28. YEGIAN, D. and BUDD, V. Am. Rev. Tuberc. 64 : 81. 1951.

ON RAT SERUM AMYLASE

I. STUDIES IN THE NORMAL AND THE ALLOXAN DIABETIC ANIMAL[1]

By Jules Tuba and G. Stuart Wiberg

Abstract

A dextrinogenic micromethod was used to establish serum amylase levels in adult male and female rats which were maintained on a standard laboratory diet. A highly significant difference was found to exist between the activities of the enzyme in the male and female rats. The effect of fasting, and of limiting food consumption, indicated a highly significant correlation between daily food intake and serum amylase levels. The polyphagia manifested by alloxan diabetic rats was not reflected in abnormally high amylase activity, as might be expected, but there was a departure from the normal response to the levels of food ingested each day. Oestradiol dipropionate and testosterone propionate were injected into normal male and female rats for seven days. Treatment with male hormone produced no significant variation in food consumption or serum amylase activity in either sex. Injections with oestradiol resulted in significantly lowered food intake in both sexes, but only in the case of males was there an accompanying fall in enzyme levels. On the basis of the experiments described in this paper it is seen that rat serum amylase consists of two fractions, and that the major portion may be considered to be of an adaptive nature. This adaptive portion appears to a large measure to be a reflection of the total daily food intake, which may be influenced by certain factors, such as sex hormones and alloxan diabetes.

Introduction

Investigations in this laboratory have been concerned for some years with alkaline phosphatase and lipase (tributyrinase) of rat serum. These studies have now been extended to rat serum amylase.

A number of factors have been shown to influence the activity of both the alkaline phosphatase and tributyrinase of rat serum. The levels of phosphatase were found to be affected by the nature and the amount of the diet consumed (1, 17, 19, 20, 22, 23), sex hormones (16), and alloxan diabetes (1). Fasting produced a decrease of enzyme activity within 24 hr. to about one quarter of the level associated with a balanced diet such as Purina Fox Checkers. These fasting levels of serum alkaline phosphatase remained unchanged for periods up to 12 days (1, 21). The variable or adaptive fraction of the enzyme in serum has been shown to be derived mainly from the intestine (8) where it appears to assist in the absorption of fat (2, 3, 8, 21). There is a highly significant correlation between serum alkaline phosphatase activity and the daily consumption of food, particularly fat (1, 14, 20). Our evidence strongly suggests that the variations in the activity of this serum enzyme associated with various dietary regimens, sex hormones, and alloxan

[1] *Manuscript received April 30, 1953.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alberta, with financial assistance from the University of Alberta Medical Research Fund and from the National Research Council, Ottawa, Canada.

diabetes may be correlated directly with the daily consumption of fat. This is supported in a rather convincing way by the fact that on a fat-free, but otherwise adequate diet, serum alkaline phosphatase levels are lowered to fasting values (14).

The responses of rat serum tributyrinase to various experimental factors proved to be more difficult to assess than in the case of serum alkaline phosphatase. Neither complete or partial deprivation of food produced any effect on the activity of the enzyme (18, 24), nor was there any correlation between food consumption and serum tributyrinase values in normal adult animals subsisting on the standard balanced laboratory diet of Purina Fox Checkers (24). However, the elevated enzyme levels in alloxan diabetic animals was found to be correlated with the greatly increased food consumption. Serum tributyrinase activity was significantly influenced by the amount (24), and nature of dietary fats (15) and fatty acids (14), as well as by the absence of fat from the diet (14). Activity of the enzyme on a fat-free diet was somewhat less than half the value observed on the standard laboratory diet, but increased towards 'normal' values as a result of fasting. Our experiments suggest that serum tributyrinase reflects physiological activities which are concerned with (1) the intestinal absorption of fatty acids and (2) the mobilization of depot fats during periods of fasting.

It is apparent that rat serum alkaline phosphatase is to a large measure an adaptive enzyme, while rat serum tributyrinase is much less responsive to dietary and experimental factors. Both enzymes remain at characteristic and constant levels during fasting for periods up to 12 days, after which there occurs a premortal fall.

There has not been reported in the literature a comprehensive study on rat serum amylase, and our studies on the other two serum enzymes are included here as a background and for comparison. This paper is concerned with a number of preliminary observations including the relationship of the enzyme to food consumption and the sex of the animals.

Experimental

Adult male or female rats were housed in individual cages and given food and water ad libitum. The standard laboratory diet of Purina Fox Checkers was fed in a powdered form in order to facilitate daily estimation of food consumption and to keep the spillage at a minimum. Body weights were recorded, usually just before the animals were bled for amylase determinations.

Blood was obtained by bleeding the animals from the tail into 3 × 70 mm. tubes. After centrifuging, the serum was removed by capillary pipettes,and 0.1 ml. was diluted at once to 2.5 ml. with 0.9% saline. In the majority of cases the diluted serum was used at once for estimation of amylase activity, otherwise it was stored in the refrigerator at 5° C. The stability of the enzyme in the stored dilute serum is shown by the fact that we have found the amylase content to be unchanged after one year.

Reagents

Starch: a 1% solution of soluble starch (Eimer and Amend, C.P.) was freshly prepared before each set of amylase determinations.

Buffer: 0.04 *M* phosphate buffer.

Stock iodine solution: 30 gm. potassium iodide in one liter 0.1 *N* iodine.

Iodine reagent: a 1 : 10 dilution of stock iodine solution, containing 5% potassium fluoride.

Amylase activity of rat serum was measured by a microadaptation of the method of Huggins and Russell (6): this required only 0.1 ml. serum. Five milliliters of the freshly prepared starch solution and 5 ml. of the phosphate buffer, pH 7.0, were equilibrated in a test tube at 37° C. for five minutes. One milliliter diluted serum, also warmed to 37° C. was added, the tube was stoppered, and the contents were mixed. One milliliter of the mixture was transferred immediately to a 100 ml. volumetric flask which contained 4 ml. dilute iodine solution and the enzyme inhibitor, fluoride. At the end of one hour, 1 ml. of the digestion mixture was added to another 100 ml. volumetric flask, containing 4 ml. of the dilute iodine reagent. The contents of the flasks were made up to 100 ml. with water and mixed. The percentage transmission of the starch–iodide solutions were read in an Evelyn colorimeter with filter 660, and a blank was used of 4 ml. dilute iodine reagent which had been made up to 100 ml. The amount of starch present at the beginning and at the end of the one hour amylolytic period was determined from a standard curve (Fig. 1), and the quantity of starch that had disappeared was then calculated.

The unit of amylase activity is that amount of the enzyme sufficient to hydrolyze 1 gm. of starch in one hour at 37° C. at pH 7.0. Not more than 50% of the substrate should be hydrolyzed during the one hour digestion period. Results are expressed as units of amylase activity per 100 ml. serum.

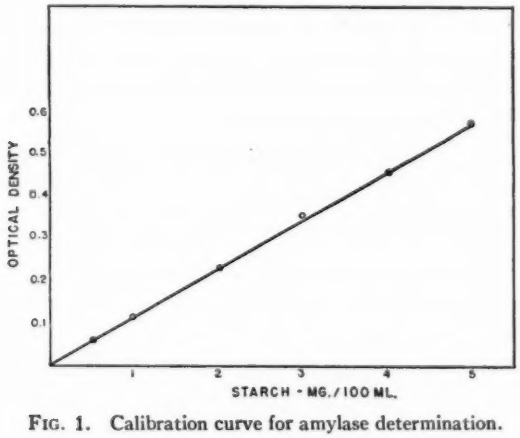


FIG. 1. Calibration curve for amylase determination.

Results

Kinetics

The kinetics of rat serum amylase do not differ significantly from the kinetics of the enzyme in human serum as reported by Huggins and Russell (6). For that reason there is only brief consideration given here to the factors which affect the action of the rat serum enzyme.

The pH activity curve of rat serum amylase for phosphate buffer showed a well-defined, fairly broad optimum, with the greatest activity at pH 6.95. This is in agreement with some of the studies on human serum amylase (10, 12, 13) although Huggins and Russell reported the optimum to be at pH 7.4. Myers, Free, and Rosinski (9) observed that the enzyme in human serum possessed a broad pH range of optimal activity, from 6.8 to 7.6. Since Sherman, Caldwell, and Adams (11) found the optimum activity of human pancreatic amylase to be at pH 7.2, Myers *et al.* decided to use that pH for their serum amylase determinations.

We obtained a Michaelis constant of 7.5 mgm. per 11 ml. for rat serum amylase. Huggins and Russell found the value for human serum amylase to be 7.14 mgm. per 10 ml. Hanes (4) obtained a Km value equivalent to 7.8–7.9 mgm. per 10 ml. for the α–amylase of sweet peas.

The action of rat serum amylase is directly proportional to time during the one hour amylolytic period used by us, and the extent of hydrolysis varies directly with enzyme concentration for the amount of serum used in our investigations.

Normal Levels of Serum Amylase in Adult Rats

Normal serum amylase activity was determined in 20 male and 20 female adult rats which had been fed ground Checkers ad libitum for seven days. Amylase values for the males were 59.7 ± 1.16 units per 100 ml. and 49.0 ± 1.10 units per 100 ml. for the females. The concentration of amylase in male rat serum is sufficient to hydrolyze approximately 60 gm. starch in one hour. This is around 25 times greater than the activity of the enzyme in human serum, and approximately the same ratio exists between the serum alkaline phosphatase activity of rats and of humans.

The difference between adult male and female rat serum amylase levels is highly significant ($t = 6.1$, $P < 0.01$). Tuba, Baker, and Cantor (16) previously noted that the same situation exists with regard to rat serum alkaline phosphatase. They also noted that the phosphatase levels varied directly with the total daily consumption of food. It is evident from their data that the influence of sex, castration, and sex hormones can be directly correlated with daily food consumption. A number of further observations in this laboratory, culminated by the experiments of Taylor, Madsen, and Tuba (14), proved that the serum phosphatase levels of rats fed a balanced diet are directly related to the daily consumption and intestinal absorption of fatty acids. In

the light of these experiences, it seemed probable that the statistically significant differences in the serum amylase levels of male and female rats might be accounted for by differences in the daily food consumption of the two sexes. It seemed necessary to find what relationship existed between daily food consumption and serum amylase levels.

The Relationship Between Food Consumption and Serum Amylase

Eight adult male rats were maintained in individual cages on an ad libitum diet of Purina Fox Checkers and water for seven days. After this stabilization period, the animals were fasted for 14 days, during which time water was always available. Blood was collected at the beginning and the end of the fast and at times indicated in Table I for determination of amylase values. Body weights were noted at each time of bleeding. Ad libitum feeding was resumed on the 15th day of the experiment and continued for 25 days, when the serum amylase values and body weights were again determined.

TABLE I

EFFECT OF FASTING ON RAT SERUM AMYLASE LEVELS AND BODY WEIGHTS

(Each value is the mean of five rats (± standard error of mean))

Fasting period, in days	Serum amylase, units per 100 ml.	Body weight, in gm.
0	49.4 ± 2.7	301 ± 9.5
1	41.6 ± 2.5	272 ± 7.5
3	35.6 ± 3.8	249 ± 6.0
7	22.3 ± 1.8	219 ± 3.9
14	12.6 ± 2.7	172 ± 3.9
—	48.3 ± 2.7*	320 ± 10.1*

* *Values for fasted animals 25 days after return to normal diet.*

The data in Table I indicate that serum amylase levels decreased during the fasting period to approximately 25% of 'normal' values by the 14th day. The decrease in activity of the enzyme is statistically significant by the third day of the fast ($t = 2.76$). Amylase values and weights of the fasted animals were restored to normal after 25 days feeding. Three of the original eight animals failed to survive the prolonged fast and results for these are not included in the data above. The serum amylase value for one of these three shortly before death was 2.0 units per 100 ml. The effect of complete food deprivation is manifested more quickly in the case of serum alkaline phosphatase, which reaches fasting levels in 24 hr. and these are maintained at constant values (1, 21) until a further sharp premortal fall.

A further experiment was devised to determine the degree of correlation between daily food consumption and serum amylase values. Ten adult male rats were fed ground Purina Fox Checkers and water ad libitum for one week.

The average daily food intake was found to be 21.0 ± 1.3 gm. After the seventh day the quantity of food placed daily before each animal was reduced to 15 gm. for three days, and this amount was decreased by 5 gm. every three days thereafter. At the end of each period on each food level values were obtained for body weights and levels of serum amylase and blood glucose (1).

TABLE II

EFFECT OF LIMITING FOOD CONSUMPTION (GM. PER DAY) ON SERUM AMYLASE LEVELS (UNITS PER 100 ML.), BLOOD GLUCOSE (MGM. %), AND BODY WEIGHTS (GM.) OF NORMAL MALE ADULT RATS

Each value is the mean of 10 rats (± standard error of mean at beginning and end of experiment)
Each food level was fed for three days before estimations were carried out

Consumption	21.0 ± 1.3	15	10	5
Amylase	55.2 ± 1.5	52.0 ± 1.6	43.5 ± 1.3	39.6 ± 1.2
Blood glucose	197 ± 12.0	123	105	77 ± 7.0
Body weight	345 ± 13.0	332	318	294 ± 10.0

The serum amylase values in Table II declined with decreasing food intake. The decreases in enzyme activity were paralleled by changes in body weight and blood sugar values. Statistical treatment of the data revealed: (1) a highly significant correlation between food consumption and serum amylase ($r = 0.77, t = 7.39, P < 0.01$), (2) a highly significant correlation between the levels of the enzyme and blood sugar ($r = 0.60, t = 4.56, P < 0.01$), and (3) a significant correlation between serum amylase and body weight ($r = 0.305, t = 2.52, P < 0.05$). It was not until the animals had been permitted to consume only 10 gm. of food daily for three days that the activity of serum amylase fell significantly below the ad libitum values ($t = 5.7$).

The Relationship Between Food Consumption and Serum Amylase of Alloxan Diabetic Rats

Our previous findings that the elevated values of serum alkaline phosphatase (1) and serum tributyrinase (18) in alloxan diabetic rats could be accounted for on the basis of increased food consumption (20, 24), which in the final analysis is correlated with the absorption and metabolism of fat (14). The correlation of serum amylase with food consumption of normal adult male rats obtained with the data of Table II suggested a similar study with alloxan diabetic animals. The experiment was repeated with 10 rats with well established alloxan diabetes, in spite of rather negative reports in the literature concerning the effect of this experimental state on the serum amylase levels of rats (5, 7). The daily ad libitum consumption of the diabetic animals, given in Table III, was significantly higher than the normal values in Table II. In spite of this, serum amylase values were not significantly different in the two experiments in the cases where the animals were permitted to eat without

restraint. There exists no correlation between food consumption and enzyme activity in the experiment with the diabetic animals. In fact, amylase activity even at the 5 gm. level of daily feeding is still statistically unchanged from the initial values. This abnormality in the diabetic animals is emphasized by the fact that at the 5 gm. level of consumption the normal animals manifest a highly significant decrease in enzyme activity compared with the ad libitum feeding ($t = 4.57$). So while it can not be said that an abnormal elevation of serum amylase exists in alloxan diabetic animals on an ad libitum diet, there is obviously a departure from the normal response to decreasing daily food intake. This doubtless is associated with altered metabolic activities of the diabetic animals. The amylase of rat serum is not likely to be associated with fat digestion and metabolism, as are the alkaline phosphatase and tributyrinase. Hence the explanation of our findings is probably associated with other dietary factors, and experiments with this in mind are in progress.

TABLE III

EFFECT OF LIMITING FOOD CONSUMPTION (GM. PER DAY) ON SERUM AMYLASE LEVELS (UNITS PER 100 ML.), BLOOD GLUCOSE (MGM. %), AND BODY WEIGHT (GM.) OF ALLOXAN DIABETIC ADULT MALE RATS

Each value is the mean of 10 rats (± standard error of mean at beginning and end of experiment) Each food level was fed for three days before estimations were carried out

Consumption	27.0 ± 1.1	25	20	15	10	5
Amylase	51.7 ± 2.8	55.6	58.0	53.6	50.2	52.6 ± 2.6
Blood glucose	434 ± 3.0	353	345	171	132	141 ± 6.6
Body weight	299 ± 9.8	296	291	285	268	248 ± 9.1

Effect of Sex Hormones on Serum Amylase in Normal Rats

It was observed above that a highly significant difference exists between serum amylase levels of normal adult male and female rats. It seemed probable that the lower activity of the enzyme in female rats might be associated with a lower daily intake of food as in the case of rat serum alkaline phosphatase (16). This relationship in normal rats due to the influence of sex hormones might therefore be emphasized following the injection of male or of female hormones.

For the first part of this experiment we used 24 adult male rats, which were divided into four groups of six. They were fed ad libitum on the usual diet in individual cages for seven days and then they were bled for serum amylase estimations. Group I was left as a control group and received no injections. Group II was injected daily with 0.2 ml. oil of sesame. Group III received daily injections of 1.0 mgm. testosterone propionate in 0.2 ml. oil of sesame. Group IV was injected daily with 0.1 mgm. oestradiol

dipropionate in 0.2 ml. oil of sesame. The injections were made subcutaneously and continued for a period of seven days. At the end of the seven day period the animals were bled again for amylase determinations. The results are presented in Table IV.

TABLE IV

EFFECT OF SEX HORMONE INJECTIONS ON NORMAL MALE ADULT RATS

Means of six animals (± standard error of the mean)

Upper figures of each pair represent values at commencement of hormone treatment, while lower figures represent values after seven days on the experiment

Group	Substance injected	Food consumption (gm.)	Body weight (gm.)	Serum amylase, units %
I	—	24 ± 1.5 21 ± 1.5	381 ± 7.6 394 ± 7.9	66.5 ± 2.0 63.1 ± 1.8
II	Oil of sesame	24 ± 1.0 17 ± 0.4	353 ± 8.7 351 ± 10.0	61.2 ± 1.4 56.5 ± 1.4
III	Testosterone	24 ± 2.2 16 ± 0.8	365 ± 14.0 360 ± 13.4	62.6 ± 2.9 56.8 ± 2.5
IV	Oestradiol	23 ± 1.20 9 ± 0.73	355 ± 13.8 319 ± 13.0	57.2 ± 1.6 47.3 ± 2.0

A slight fall of nearly ten per cent was found in the enzyme level of animals of Group III which were injected with testosterone, and this was approximately the same as the decrease which occurred in the rats injected with oil of sesame (Group II). This may be attributed to the slightly decreased food intake which in these two groups is more marked than in the control animals of Group I. There was no significant weight loss in spite of decreased consumption, so it may be assumed that the oil of sesame compensated to a large extent for the caloric losses involved. The injection of oestradiol in Group IV produced the most striking results. Enzyme activity fell by 17% while food intake decreased by 60%, and there was an average weight loss of 35 gm. in seven days. A statistical analysis of serum amylase values for Group IV indicates that the effect of the injected female sex hormone produced decreases in enzyme activity which are highly significant ($t = 5.0$, $P < 0.01$), whereas the differences in the other groups are not significant. The correlation between food intake and amylase levels in this group is highly significant ($r = 0.838$, $t = 4.81$, $P < 0.01$) as was the case with the data of Table II.

The second part of this experiment was the same as the first except that female rats were used instead of males. The results are reported in Table V.

TABLE V

EFFECT OF SEX HORMONES ON NORMAL VIRGIN FEMALE ADULT RATS

Means of six animals (± standard error of the mean)
Upper figures of each pair represent values at commencement of hormone treatment, while lower figures represent values after seven days on the experiment

Group	Substance injected	Food consumption (gm.)	Body weight (gm.)	Serum amylase, units %
I	—	17 ± 1.4 15 ± 1.5	198 ± 3.3 202 ± 6.0	49.2 ± 1.0 55.0 ± 4.3
II	Oil of sesame	17 ± 0.6 15 ± 0.7	197 ± 1.9 205 ± 1.6	49.9 ± 2.8 53.8 ± 2.6
III	Testosterone	16 ± 1.1 15 ± 0.7	195 ± 3.5 212 ± 1.6	45.4 ± 0.8 52.7 ± 3.6
IV	Oestradiol	16 ± 0.6 10 ± 0.3	201 ± 2.5 188 ± 0.8	45.0 ± 0.5 43.4 ± 0.5
		20 ± 0.8* 15 ± 1.1**	198 ± 2.9* 208 ± 1.2**	61.2 ± 2.7* 54.2 ± 2.4**

* *Values seven days after cessation of oestradiol injections.*
** *Values 14 days after cessation of oestradiol injections.*

There were no significant changes in values of food consumption, body weight, or serum amylase in the first three groups. The amount of food eaten by control female rats was significantly lower than for male controls (Group I in Table IV and in Table V) ($t = 3.60$, $P < 0.01$). Oestradiol reduced food consumption very profoundly, and to about the same values as in Table IV. There was once more an accompanying drop in weight although not to the same degree as with the males. There were no significant alterations in the enzyme levels produced by oestradiol although a comparison of the four groups indicates a lower trend in Group IV. Weights in Group IV returned to normal after injections were discontinued. Food consumption and enzyme levels were abnormally high seven days after cessation of oestradiol treatment, but after 14 days these had once more returned to values characteristic of female rats. The lack of correlation between serum amylase and food consumption contrasts markedly with the results for males.

Experiments similar to those recorded in Tables IV and V have been begun with castrated males and ovariectomized female rats. It is hoped that they will show more clearly the relationship between serum amylase levels, food consumption, and sex hormones.

References

1. CANTOR, M. M., WIGHT, P. A., and TUBA, J. Trans. Roy. Soc. Can. V, 42 : 51. 1948.
2. FLOCK, E. V. and BOLLMAN, J. L. J. Biol. Chem. 184 : 523. 1950.
3. GOULD, B. S. Arch. Biochem. 4 : 175. 1944.
4. HANES, C. S. Biochem. J. 26 : 1406. 1932.
5. HECHT, E. Biochim. et Biophys. Acta, 1 : 415. 1947.

6. HUGGINS, C. and RUSSELL, P. S. Ann. Surg. 128 : 668. 1948.
7. LUKENS, F. D. W. Physiol. Revs. 28 : 304. 1948.
8. MADSEN, N. B. and TUBA, J. J. Biol. Chem. 195 : 741. 1952.
9. MYERS, V. C., FREE, A. H., and ROSINSKI, E. E. J. Biol. Chem. 154 : 39. 1944.
10. O'DONOVAN, D. and DAVISON, W. C. Bull. Johns Hopkins Hosp. 40 : 238. 1927.
11. SHERMAN, H. C., CALDWELL, M. L., and ADAMS, M. J. Am. Chem. Soc. 50 : 2529. 1928.
12. SHERMAN, H. C., THOMAS, A. W., and BALDWIN, M. E. J. Am. Chem. Soc. 41 : 231. 1919.
13. SOMOGYI, M. J. Biol. Chem. 134 : 301. 1940.
14. TAYLOR, J. D., MADSEN, N. B., and TUBA, J. Can. J. Med. Sci. 30 : 308. 1952.
15. TAYLOR, J. D. and TUBA, J. Can. J. Med. Sci. 30 : 453. 1952.
16. TUBA, J., BAKER, D. B., and CANTOR, M. M. Can. J. Research, E, 27 : 202. 1949.
17. TUBA, J., CANTOR, M. M., and RICHARDS, A. G. Can. J. Research, E, 27 : 25. 1949.
18. TUBA, J. and HOARE, R. Can. J. Med. Sci. 29 : 25. 1951.
19. TUBA, J., JELINEK, B., SHAW, R. K., and MADSEN, N. B. Can. J. Med. Sci. 30 : 378. 1952.
20. TUBA, J. and MADSEN, N. B. Can. J. Med. Sci. 30 : 18. 1952.
21. TUBA, J. and ROBINSON, M. I. J. Biol. Chem. In press.
22. TUBA, J. and SHAW, R. K. Can. J. Research, E, 28: 41. 1950.
23. TUBA, J., SILUCH, K. A., ROBINSON, M. I., and MADSEN, N. B. Can. J. Med. Sci. 30: 515. 1952.
24. TUBA, J. and TAYLOR, J. D. Can. J. Med. Sci. 30 : 26. 1952.

A METHOD FOR THE DETERMINATION OF THE EFFECTS OF DRUGS ON THE PAIN THRESHOLD OF HUMAN SUBJECTS[1]

By G. A. Deneau,[2] R. A. Waud, and C. W. Gowdey

Abstract

A method is presented for determining the effects of analgesic agents upon the pain thresholds of human subjects. The method consists of inducing a sharp pain in the calf muscles by applying pressure with a sphygmomanometer cuff; the pressure required to induce pain is taken as a measure of the pain threshold. By controlling a few variables in the procedure, the normal threshold can be determined with an accuracy of ±10 mm. Hg pressure. With placebos, a maximum increase of 4% over the normal pain threshold occurred one hour after administration. Acetylsalicylic acid produced an increase of approximately 20% at the same period; this was significantly greater than placebo effects. The effect of mild narcotic agents, given orally, upon the pain threshold was also measured. Codeine phosphate and pethidine hydrochloride both produced a significant increase in the pain threshold. The method presented permits the use of untrained human subjects and is sufficiently sensitive to measure the effects of the antipyretic analgesics.

Introduction

Many compounds are currently being synthesized in an effort to find potent, yet safe, analgesic agents. The appearance of these compounds demands suitable methods for evaluating their effect upon the human subject. If large clinical facilities are available, these new drugs can be tested on patients actually suffering from pain, but since such facilities are not usually available, normal subjects must be used. With all methods which employ normal subjects, an elevation in the pain threshold is considered to be indicative of analgesia; hence, analgesic drugs are compared by the extent to which they cause the pain threshold to rise. Several excellent reviews (3, 8, 9) have been written which discuss the algesimetric procedures currently employed. In all these methods, a noxious stimulus is applied to some accessible site on the body to elicit pain, which the subject usually reports verbally. To date, many of these studies cannot be verified because the stimuli used produce more than one sensation, so that only highly trained subjects can discriminate the onset of pain. It is then especially difficult to measure the low grade effects of the milder analgesic agents.

A method is advanced in the present paper which permits the use of untrained subjects. The method was developed to measure the effects of the antipyretic analgesics. Muscular pain, which is especially amenable to this type of agent (4), is produced in the calf muscles of the leg. The pain threshold is measured as the pressure in a sphygmomanometer cuff which is required to produce this pain (1, 2).

The apparatus consists of a mercury manometer capable of registering pressures up to 750 mm. Hg. This is attached to a standard 'hook-on' sphygmomanometer cuff which is inflated by a foot-pump.

[1] *Manuscript received March 30, 1953.*
Contribution from the Department of Pharmacology, University of Western Ontario, London, Canada.
[2] *Present address: Department of Pharmacology, University of Michigan, Ann Arbor, Michigan.*

Highly intelligent, healthy, human subjects between the ages of 20 to 35 years were used in this study. None showed any signs of peripheral vascular disease nor reported any history of allergy to analgesic drugs. Acetylsalicylic acid (20 grains), codeine phosphate (½ grain), and pethidine hydrochloride (1½ grains) were all given orally. Citrate flavored and nonflavored starch placebos were also administered. Neither the subjects nor the operator were aware of the nature of the drugs, except by code number, during the experiment. The subjects were not permitted to see the manometer. Changes in pain threshold are expressed as percentage changes from normal. Statistical evaluation of the data is based upon methods described by Snedecor (10) for analysis of grouped data; "*P*" values of 0.05 or less being considered significant.

The subject is seated comfortably in a chair, with the leg partly flexed, so that both the heel and the ball of the foot rest on the floor and the angle of the knee joint is approximately 135 degrees. Since the threshold varies with the degree of extension of the leg (increasing with flexion and decreasing with extension) it is necessary to maintain the same degree of extension for all measurements of pain threshold. This is easily accomplished by placing the subject's heel on a line or against a block which is at a fixed distance from the chair. After the cuff is placed snugly about the calf of one leg, it is inflated at a rate of 40 mm. Hg pressure per second. Inflation is discontinued and the pressure immediately released when the subject reports the onset of pain. The pressure at this point, as read from the manometer, is recorded as a measure of the pain threshold. After a period of at least 30 sec., the cuff is re-inflated and another reading of pain threshold recorded. If the onset of pain has been sharp and definite, repeated determinations of this sort usually agree within 10 mm. Hg pressure. If, however, definite onset and close agreement are not achieved, slight adjustment of the cuff up or down the leg may be necessary before the pain arises clearly and sharply. The position of the cuff is then marked on the leg so that the cuff may be reapplied in the same position for subsequent determinations of threshold. Thresholds are recorded to the nearest 10 mm. Hg pressure. This preliminary determination of a suitable position for the cuff is sufficient to acquaint a new subject with the nature of the end point and no further training is necessary.

Immediately before the drug is administered to the subject, the normal pain threshold is determined. The drugs can be given by any desired route and thresholds are then determined at appropriate intervals. In the present study, the drugs were all given orally and thresholds determined at periods of 30, 60, and 120 min. after administration.

Results

Fig. 1 shows the frequency distribution of 561 normal pain thresholds determined on 177 subjects, the mean threshold being 310 mm. Hg. Although there is some skewing to the right, the curve meets all tests for normalcy. Of these values, 128 normal thresholds were determined on 32 female subjects,

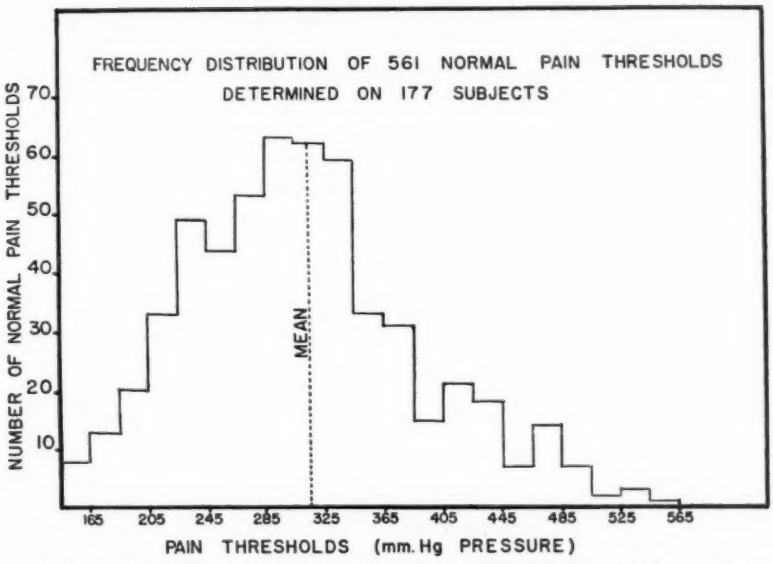


FIG. 1. The frequency distribution of the normal pain thresholds which were determined prior to the administration of drugs and placebos. The mean threshold is 310 mm. Hg pressure.

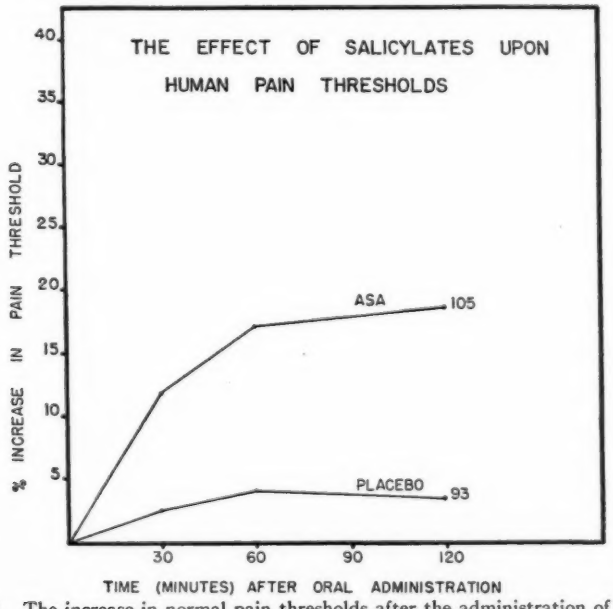


FIG. 2. The increase in normal pain thresholds after the administration of placebos, and after 20 grains acetylsalicylic acid (ASA). The figures to the right of each curve represent the number of subjects upon whom the mean values were determined.

with a mean normal threshold of 300 mm. Hg pressure, suggesting that the responses were essentially similar for the two sexes. Fig. 2 illustrates the effects of placebos and a salicylate compound upon the pain thresholds. Although placebos produced a slight effect (significant elevation of 4% at 60 min.), the acetylsalicylate showed significantly greater effects at all periods measured. No subject receiving a placebo showed a change in threshold at any time which was as great as the mean effect of acetylsalicylic acid. Table I summarizes the statistical evaluation of these results.

TABLE I

COMPARISON OF THE EFFECTS OF ACETYLSALICYLIC ACID (ASA) (20 GRAINS) AND PLACEBOS (P)

Drugs	Doses, grains	No. of subjects	Time after administration, min.	$\bar{n}$ (mean diff.)	"P"
ASA	20	102	30	9.3	$\ll 0.01$
P	—	93	60	13.1	$\ll 0.01$
			120	15.2	$\ll 0.01$

$\bar{n}$ — Difference between the mean responses of two drugs at a given time.
P — The probability that $\bar{n}$ is due to chance.

Fig. 3 illustrates the effects of codeine phosphate and pethidine hydrochloride upon the pain threshold. Codeine was the more effective at 30 min. but there was no significant difference between the effects of these two drugs at 60 and 120 min. Some of the subjects in this latter study exhibited overt depression while others showed a release of inhibition, and others seemed to have no mental disturbances. Although no attempt was made to quantitate these various changes in mental attitude, there was no apparent correlation between these changes and effects upon the pain threshold.

Discussion

Although strong analgesic agents were not employed in the present study, the results obtained with agents of mild and moderate potency indicate that this method is suitable for measuring the effects of drugs upon the pain threshold. The threshold elevation produced by acetylsalicylic acid is comparable to that shown by other methods of measuring pain threshold (5, 6). In this study, however, the effect of placebos, though significant, is slight and significantly less than that of common pain-relieving agents. As with other methods, the stimulus used in this procedure at first gives rise to a nonpainful sensation. However, the change in modality from pressure to pain is not a graded effect, but rather a sharp transition making it possible to discriminate the end point with minimal previous training. The myalgia is a deep pain which more closely resembles the type of pain for which analgesic agents are

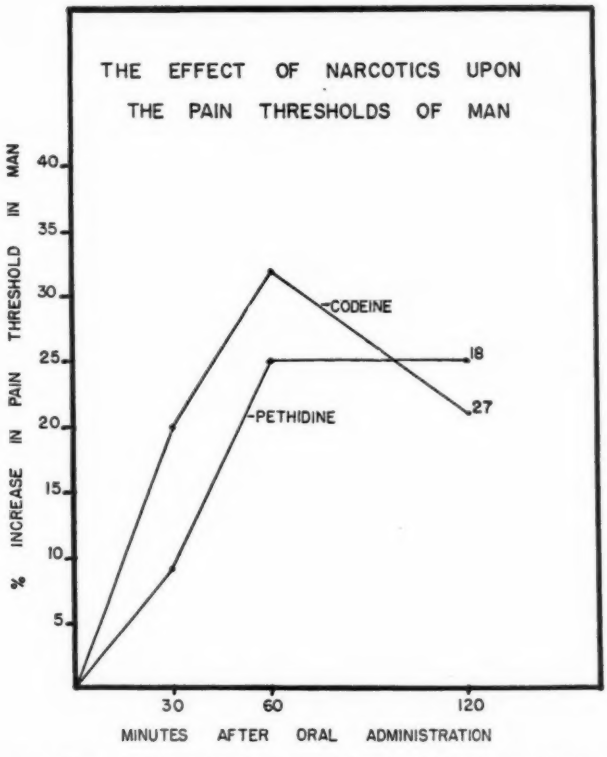


FIG. 3. The effects of codeine phosphate (½ grain) and pethidine hydrochloride (1½ grains) upon the normal pain threshold, using the placebo curve of Fig. 2 as baseline. The figures to the right of each curve represent the number of subjects upon whom the mean values were determined.

employed than does the cutaneous pain which is employed in some of the other currently used algesimetric methods. Since the subjects had had very little training, the low placebo effects illustrate two points: (1) the ease of discriminating the threshold of pain in this procedure by intelligent, but untrained subjects, and (2) the rapidity with which the pain increases upon further stimulation (a stimulus increase of 10% beyond the threshold of the untreated subject causes an intolerable pain of cramping nature).

The fact that codeine and pethidine showed little more effect than acetylsalicylic acid need not cause great concern when two factors are taken into account: the absorption of the former compounds when taken orally is unpredictable, and elevation of the pain threshold is not the only factor involved in the relief of pain. Relief of pain is also dependent upon relief of anxiety, fear, and apprehension. The 'narcotic' analgesics produce this latter effect by inhibiting the patient's powers of attention, particularly toward distressing

elements, either physical or mental. No method yet devised measures these two mechanisms simultaneously. Hill *et al.* (7) have devised a unique method for measuring a drug's ability to allay anxiety. A method such as this, used in combination with the method described in this paper, would provide a more complete measurement of the pain-relieving properties of analgesics which produce a pronounced effect on the cerebral cortex.

Summary

The effect of analgesic drugs upon the pain threshold of man can be evaluated by determining the pressure required to elicit a deep muscular pain in the calf of human subjects. Very little training is necessary for subjects to recognize the pain threshold. Factors which influence the normal pain threshold and which must be controlled rigidly are as follows:

(1) The degree of extension of the leg must be the same for all determinations of threshold in a given experiment.

(2) The cuff must be reapplied to the leg at the same level for all determinations in a given experiment. Changes in the position of the cuff of as little as one inch can cause the threshold to vary up to 25%.

(3) Strenuous exercise causes an increase in the normal pain threshold; hence, undue activity on the part of the subject must be restricted.

Acetylsalicylic acid produces an increase in the pain threshold which is significantly greater than that resulting from placebo administration.

Suggested Refinements in the Method

Since the bulk of the studies reported herein have been completed, three new features have been incorporated into the method which improve its reliability. Compressed air from the laboratory air line or from a tank is used to inflate the cuff. A filling pressure of 15 lb. has been found satisfactory when a length of capillary tubing is inserted into the intake line to regulate the rate of inflation at 40 mm. Hg per second. By means of a two-way valve, the subject controls the inflation of the cuff to the point of pain. At this point, he releases the valve and the pressure is released from the cuff. The insertion of a one-way valve in the line leading to the manometer causes the mercury to remain at the point of maximum pressure after the cuff has been deflated. This permits the operator to make a more accurate reading of the pain threshold.

Acknowledgments

The authors wish to express their appreciation to Reckitt and Colman (Canada) Limited, who supported part of this research. Many thanks are also due to those who so kindly gave their time in the capacity of experimental subjects.

References

1. DENEAU, G. A., WAUD, R. A., and GOWDEY, C. W. Rev. can. biol. 11 : 59. 1952.
2. DENEAU, G. A. and GOWDEY, C. W. Federation Proc. 11 : 338. 1952.
3. GOETZL, F. R., BURRILL, D. Y., and IVY, A. C. Quart. Bull. Northwestern Univ. Med. School, 17 : 280. 1943.
4. GOODMAN, L. and GILMAN, A. The pharmacological basis of therapeutics. The MacMillan Co., New York, 1941. p. 227.
5. HARDY, J. D., WOLFF, H. G., and GOODELL, H. J. Clin. Invest. 19 : 649. 1940.
6. HARRISON, I. B. and BIGELOW, N. H. Research Pubs. Assoc. Research Nervous Mental Disease, 23 : 154. 1943.
7. HILL, H. E., KORTENSKY, C. H.; FLANARY, H. G., and WIKLER, A. Arch. Neurol. Psychiat. 67 : 612. 1952.
8. MILLER, L. C. Ann. N.Y. Acad. Sci. 51 : 34. 1948.
9. PFEIFFER, C. C., SONNENSCHEIN, R. R., GLASSMAN, L., JENNEY, E. H., and BOGOLUB, S. Ann. N.Y. Acad. Sci. 51 : 21. 1948.
10. SNEDECOR, G. W. Statistical methods applied to experiments in agriculture and biology. Iowa State College Press, Ames, Iowa. 1946.

THE COMBINATION OF HEPARIN AND BOVINE SERUM ALBUMIN[1]

By D. W. Clarke and F. C. Monkhouse

Abstract

The complexing of heparin and bovine serum albumin in acetate buffer at pH 5.0 was studied by electrophoretic techniques. A stable complex with a heparin : albumin mole ratio of 1 : 2 was found, though there was evidence for the existence of unstable complexes with a higher heparin : albumin ratio.

Introduction

In the course of some studies on the relationship of heparin to plasma proteins, we have investigated a simple system containing heparin and bovine serum albumin (hereafter referred to as BSA). The effect of proteins on heparin activity has been previously studied by Jaques (3) and Fischer (1), who showed that heparin combined strongly with many proteins, and that its activity was influenced by this combination. Gorter and Nanninga (2) have studied the heparin–BSA system by precipitation of a heparin–albumin complex, followed by electrophoretic analysis of the dissociated complex. They found a heparin : BSA mole ratio of approximately unity in complexes formed at pH 1. In this study we have fractionated mixtures of heparin and BSA at pH 5 by electrophoresis-convection, and an analysis of the fractions obtained has shown that a strong combination of heparin and BSA can take place. A stable complex of one heparin molecule and two BSA molecules may form, if sufficient heparin is present in the system. With smaller amounts of heparin, other complex species are present. A similar investigation of the BSA-desoxypentosenucleic acid system has recently been described by Singer, Timasheff, and Kirkwood (7).

The theoretical equation describing the transport of two noninteracting substances in the electrophoresis-convection cell has been worked out by Kirkwood *et al.* (4).

[1]
$$f^{\frac{1}{\beta}} = \frac{1 - \chi(f - 1)}{\gamma}$$
$$f = (c_2/c_2^0)/(c_1/c_1^0)$$
$$\gamma = (c_1 + c_2)\,(c_1^0 + c_2^0)$$
$$\chi = (c_2/c_1^0 + c_2^0)$$
$$\beta = 1 - (\mu_2/\mu_1)$$

C_1^0, c_2^0 are the initial concentrations of the two components in the upper chamber of the cell.

C_1, c_2 are the final concentrations of the two components in the upper chamber of the cell.

μ_1, μ_2 are the electrophoretic mobilities of the two components. In the studies with BSA and desoxypentosenucleic acid (*vide supra*) it was shown that, under conditions of complexing, agreement between theoretical and observed fractionation ratios could only be obtained if the mobility values used for the

[1] *Manuscript received May 27, 1953.*
Contribution from Department of Physiology, University of Toronto, Toronto, Ont.

determination of β were not those measured in a Tiselius apparatus for free BSA or free desoxypentosenucleic acid, but rather those of observed complexes of different mobility. In this work, we have used the observed values of "f", the fractionation ratio, to determine β and hence of μ_2/μ_1. From these values, the presence of more than one type of complex may be deduced.

Experimental

A solution of 1% BSA was made in acetate buffer, pH 4.7, $\Gamma/2 = 0.05$. At this pH near the isoelectric point of the BSA, there should be little movement of this component out of the top cell as the experiment progresses, if there is no complexing with heparin. To 40 ml. portions of this solution, various quantities of heparin were added, and the mixture was dialyzed for 12 hr. against 1 liter of buffer. The solution was then placed in the electrophoresis-convection cell in a cold room, and fractionation was allowed to proceed for seven hours. Samples of $1\frac{1}{2}$ ml. were removed from the upper chamber at one, two, three, and four hours after the start of the run. At the end of the run, the top and bottom fractions were separated. Owing to electrolysis of the buffer, the solution at the end of the run was at pH 5.0. Total nitrogen determinations were made on each sample by the microKjeldahl method, and heparin was determined by the method of Monkhouse and Jaques (5).

Similar mixtures of heparin and albumin at pH 5.0 were made up for Tiselius electrophoretic analyses. These analyses were performed in an American Instrument Company apparatus at 2° C.

Results

The results of several runs in the electrophoresis-convection cell are shown in Table I. Concentrations of albumin and heparin at intermediate times are not given.

TABLE I

RESULTS OF FRACTIONATION OF MIXTURES OF HEPARIN AND BSA BY ELECTROPHORESIS-CONVECTION

Initial albumin concentration (mgm./ml.), A_0	Final albumin concentration, A_7	Initial heparin conc. (mgm./ml.), H_0	Final heparin conc., H_7	Initial moles heparin / Initial moles albumin H_0/A_0	Mobility ratio μ_2/μ_1 (calculated)
8.63	7.25	0.044	0.030	0.020	2.19
8.90	7.44	0.072	0.052	0.032	1.81
14.50	10.62	0.22	0.12	0.061	1.95
8.45	6.42	0.22	0.16	0.10	1.16
8.01	6.08	0.28	0.18	0.14	1.60
10.30	5.55	0.54	0.17	0.21	1.87
8.33	4.96	0.45	0.20	0.22	1.57
8.47	0.36	1.05	0.08	0.50	0.82
7.79	0.63	1.10	0.10	0.56	0.95
8.58	0.34	2.10	0.08	0.98	1.01
7.96	0.28	2.80	0.18	1.41	0.81

NOTE: *Molecular weights of BSA and heparin are taken to be 68,000 and 17,000, respectively.*

From the initial and final concentrations of heparin and of BSA, there may be calculated the various parameters of equation (1), and so a calculated value for the ratio of the mobilities of the two components may be obtained. This calculated value is shown in the last column on Table I.

By plotting $(1 - A_7/A_0)$, and $(1 - H_7/H_0)$ against H_0/A_0, curves, as shown in Fig. 1, are obtained. At the point where the curve begins to flatten

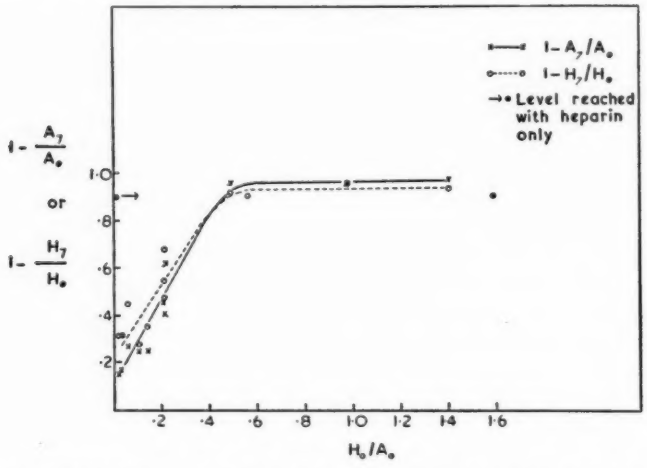


FIG. 1. Fractional amount of heparin or BSA removed from top chamber, plotted against initial mole ratio of heparin to BSA.

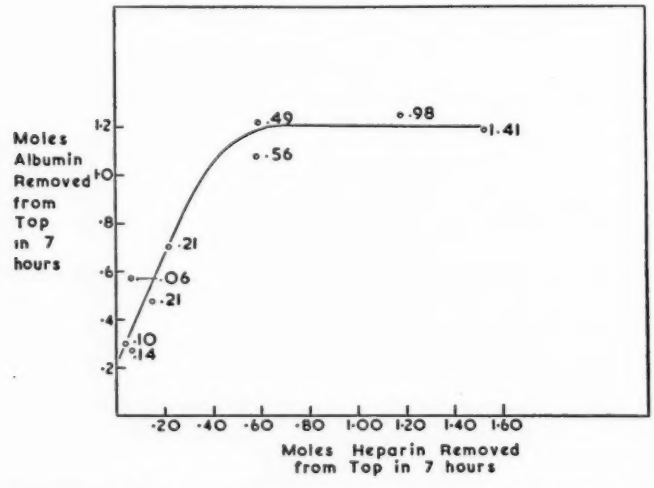


FIG. 2. Moles of heparin removed from top chamber, plotted against moles of BSA removed from top chamber, with the different initial mole ratios shown at plotted points.

out, the molecular ratio of $H_0/A_0 = \frac{1}{2}$. At this point, the rate of heparin transport out of the top cell is approximately the same as that for heparin alone. This is also indicated in Fig. 2, which shows that the amount of albumin which is removed, per mole of heparin which is removed, reaches a maximum and constant value when two moles of BSA are being removed per mole of heparin.

Separate electrophoretic analyses with a Tiselius apparatus on various mixtures of heparin and albumin showed that different components, with different mobilities, were present. The results are summarized in Table II.

Mobilities as measured in the descending limb of the Tiselius cell are used, as the conditions in this limb will approximate most closely the conditions of transport in the electrophoresis-convection cell.

TABLE II

MOBILITIES OF COMPONENTS APPEARING IN VARIOUS MIXTURES OF HEPARIN AND BSA

Moles heparin / Moles BSA	Mobilities of components (descending boundaries), $cm.^2 sec.^{-1} volt^{-1} \times 10^5$	Mobility ratio μ_2/μ_1 (measured)
BSA only	1.3	
0.17	1.8 3.0* 4.2**	1.7
0.34	5.7 9.0	1.6
0.69	11.6 23.7	0.49
1.50	12.5 22.7	0.55
Heparin only	20.8	

* *Major component.*
** *Very small component.*

Figs. 3 and 4 show the electrophoretic patterns obtained from two of the mixtures. The significance of these patterns is discussed below.

Discussion

If transport of the two components had occurred without interaction, the calculated value of the mobility ratio should agree with the observed value of μ_2/μ_1. It is seen that the calculated value is actually far below this. This lower value may be explained on the basis of complexing, i.e. the fractionation is not taking place between heparin and BSA, but rather between some complexes of the two of intermediate mobility.

A consideration of the experimental data leads us to conclude that under the conditions of this experiment, heparin and albumin can combine to form a stable complex in which the molecular ratio of heparin to BSA is 1 : 2. Our reasoning is as follows:—

1. Fig. 2 shows the amount of BSA which is removed from the top chamber in seven hours, plotted against the amount of heparin which is removed from the top chamber in the same time. The various values of H_0/A_0 are also shown, and it is seen that, above a certain value, the amount of BSA which is transported out is constant, whereas the heparin transport continues to increase. We consider that the H/A ratio at this critical point represents a situation where, on the average, one albumin molecule is holding in a stable complex, all of the heparin molecules which it can hold under these conditions. Further addition of heparin only results in the appearance of more free heparin in the solution. The analysis of mixtures in the Tiselius apparatus tends to confirm this view. The fast-moving peak in Fig. 4 (which corresponds to a mixture in which the initial heparin/BSA ratio was 1.5) has essentially the same mobility as free heparin. This peak is absent from the pattern of Fig. 3 (which corresponds to an initial heparin/BSA ratio of 0.17).

2. The fact that both heparin and BSA are transported at a constant fractional rate after the addition of a certain amount of heparin has been added, as shown in Fig. 1, indicates that there is no formation of stable complexes of a higher heparin/BSA ratio, nor is there any increase in concentration of an existing complex. This means that additional heparin must be present in an uncomplexed form. The possibility of the existence of unstable complexes of higher heparin/BSA ratios is not excluded, on the basis of this evidence, but these, if they could dissociate in a short time relative to the duration of the experiment, would act as a stable complex and free heparin. That such complexes do form, in fact, is shown by a consideration of Fig. 4. The clean separation of the heparin component from the complex in the ascending pattern and the presence of intermediate components in the descending pattern, as shown by the elevation of the pattern above the base line, in the region between the two peaks, indicate the formation of readily dissociable complexes (6).

3. The fact that the mobilities of the components reach essentially constant values, after increasing rather quickly, as more heparin is added to the solution (Table II), corroborates the conclusion that no stable complexes are being formed after the critical point has been reached.

4. A consideration of the calculated mobility ratios, as found in Table I, is of interest. It is seen that these calculated ratios decrease as the heparin/BSA ratio is increased, from values which are greater than unity, to values which are slightly less than unity. For the calculation, component (2) was assumed to be heparin, while component (1) was assumed to be albumin. Actually, the two components are different complexes but component (2) has a higher heparin/BSA ratio, and hence a higher mobility, than component (1). From Table II, the increase in mobility of these components with increasing heparin/BSA ratios may be noted. As more heparin is added to the

PLATE I

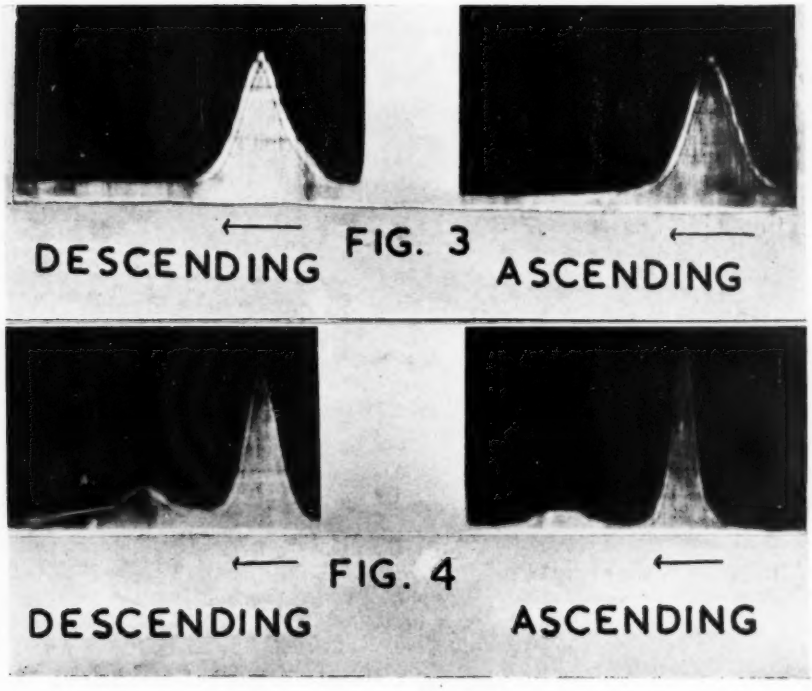


FIG. 3. Electrophoretic pattern of heparin–BSA mixture. $H_0/A_0 = 0.17$.
FIG. 4. Electrophoretic pattern of heparin–BSA mixture. $H_0/A_0 = 1.50$.

system, slow-moving complexes are changed to the more rapidly-moving complexes, and the value of the mobility ratio approaches unity. Actually, there may be more than two complexes present at one time, and as the relative amounts of the complexes, with their different mobilities, change with different experimental conditions, the calculated mobility ratios tend to vary, rather than to assume certain fixed values.

However, beyond a certain point, the calculated ratios become less than unity. This must mean one of two things:

(*a*) Component (1), the heparin-poor component, has attained a greater mobility than the heparin-rich component. This seems very unlikely, from physical considerations.

(*b*) The alternative is to assume that the fractionation is now taking place between new components, and that the faster component, which is probably the heparin-rich component, is now the component whose mobility appears in the denominator of the ratio. As previously shown, the Tiselius patterns indicate that a new, rapidly-moving species (uncomplexed heparin) has now appeared.

Acknowledgments

The heparin used in these experiments was donated by the Connaught Medical Research Laboratories, Toronto. The authors wish to acknowledge the technical assistance of Miss H. L. Dickie and Miss J. A. Fulton. The work was supported in part by grants from the National Research Council and the Defence Research Board.

References

1. FISCHER, A. Biochem. Z. 249 : 364. 1931.
2. GORTER, E. and NANNINGA, L. Proc. Koninkl. Nederland. Akad. Wetenschap., Ser. C, 55(3) : 341. 1952.
3. JAQUES, L. B. Biochem. J. 37 : 189. 1943.
4. KIRKWOOD, J. G., CANN, J. R., and BROWN, R. A. Biochim. et Biophys. Acta, 5 : 301. 1950.
5. MONKHOUSE, F. C. and JAQUES, L. B. J. Lab. Clin. Med. 36 : 782. 1952.
6. SINGER, S. J. and CAMPBELL, D. H. J. Am. Chem. Soc. 74 : 1794. 1952.
7. SINGER, S. J., TIMASHEFF, S. N., and KIRKWOOD, J. G. J. Am. Chem. Soc. 74 : 5985. 1952.

THE EFFECT OF AGE, SEX, AND PREVIOUS PREGNANCY ON OXYGEN UPTAKE OF MAMMARY TISSUE OF MICE OF THE C_{57}BL, A, AND C_3H STRAINS[1]

By Jules Tuba, Herbert E. Rawlinson, Lorna G. Shaw, M. Shirley Fraser, and Irmgaard Jeske

Abstract

Oxygen uptake of mammary tissue of mice of the C_{57}BL, A, and C_3H strains has been studied in the presence ($Q_{P^2}^{O_2}$) and in the absence of *p*-phenylenediamine (Q_{O_2}). In general, these values are affected significantly by the age, sex, breeding history, and strain of the animals. The effect of these factors on enzyme activity has been observed with a number of enzyme systems and once more emphasizes their importance in evaluating data obtained in animal enzyme studies. A notable departure from the usual pattern for the Q_{O_2} values was observed with mice of the C_3H strain. There was a significant rise in the Q_{O_2} values and a diminution in the cytochrome reserves ($Q_{P^2}^{O_2} - Q_{O_2}$) for breeder females about the time spontaneous mammary tumors begin to appear. A similar elevation in Q_{O_2}, and diminution in reserves of the cytochrome system, appeared in mammary tumor tissue obtained from C_3H females. The situation for virgin females is much the same with regard to the Q_{O_2} and $Q_{P^2}^{O_2} - Q_{O_2}$ values of mammary tissue. The males of this strain manifest an abnormal elevation in Q_{O_2} values but there is no diminution in $Q_{P^2}^{O_2} - Q_{O_2}$ levels.

Introduction

Rawlinson and Pierce have shown striking differences in the histologically demonstrable iron and in the total iron content of murine mammary tissue. Variations in iron content were found to be associated with sex, pregnancy, lactation, and strain differences (3, 4). These same experimental factors have been shown to influence serum tributyrinase (6) and serum alkaline phosphatase levels (7) in mice of the C_{57}BL, A, and C_3H strains. Age, sex, and previous pregnancy were found to influence the oxygen consumption of rat mammary tissue slices (8). It seemed probable that the same factors might affect the activity of the iron-containing cytochrome system in the breast tissue of inbred mice, and that susceptibility to mammary cancer might be reflected in the levels of oxidative activity.

Craig, Bassett, and Salter (1) have noted that the oxidative behavior of tumor tissue is abnormal and response to added *p*-phenylenediamine or succinate is decreased. They have interpreted their results as evidence of decreased concentration of the iron-containing cytochrome system in the neoplasms studied by them, and these included spontaneous mammary carcinomas of inbred strains of mice. Greenstein (2) has studied oxidation by various tissues in the presence of *p*-phenylenediamine when an excess of cytochrome *c* is added. His observations have led him to conclude that malignant tissues can be differentiated from normal tissues on the basis of cytochrome content.

[1] *Manuscript received May 28, 1953.*

Contribution from the Departments of Biochemistry and Anatomy, University of Alberta, Edmonton, Alberta. The investigation was supported by grants from the National Cancer Institute of Canada.

We have already reported the effect of age, sex, and previous pregnancy on oxygen uptake of rat mammary tissue in the presence and the absence of *p*-phenylenediamine (8, 9). These studies have now been extended to mice of the C_3H, A, and $C_{57}BL$ strains.

Experimental

The mice used in this investigation were of the C_3H, A, and $C_{57}BL$ strains. Their ages varied from 8 to about 60 weeks. Animals were housed in groups of about six in metal cages, and they were given food and water ad libitum. Breeder females were segregated for about four weeks after they had weaned their last litters, to ensure the use of resting mammary gland tissue.

Manometric techniques were similar to those used for our studies with rat mammary tissue (9). Because the quantity of tissue is smaller in the mice, the two inguinal glands of each animal, following the excision of the lymph nodes, were sliced and combined to ensure measurable oxygen consumption readings. The values of endogenous oxygen uptake (Q_{O_2}) for murine mammary tissue as well as oxygen uptake in the presence of *p*-phenylenediamine ($Q_P^{O_2}$) were calculated in μl./mgm./hr. on the basis of dry, fat-free weight. Oxygen consumption both in the presence and absence of *p*-phenylenediamine was completely inhibited by 0.003 *M* sodium cyanide.

Results and Discussion

Mice of the three strains were divided into various age groups and the values of Q_{O_2}, $Q_P^{O_2}$, and $Q_P^{O_2} - Q_{O_2}$ obtained with mammary tissue of males, virgin females, and breeder females are given according to age in Tables I, II, and III. Statistical analyses of the three tables indicate that there are definite effects of age, sex, and breeding associated with the experimental values. Because of the complexity of the situation, statistical analyses of the tables are not included, but levels of significance that are mentioned below vary between $P < 0.01$ and $P < 0.05$.

There is no significant effect of age on endogenous oxygen uptake of mammary tissue of males of the $C_{57}BL$ and A strains, but the variations in the values for the C_3H males are significant. This correlates with the findings of Richardson (5) that strain differences exist in the size and structure of the mammary glands of male mice. There are also differences in iron content (Rawlinson, unpublished data). There appears to be a definite trend in females of the three strains associated with age and breeding, similar to that noted with female rats (8). There are exceptions to be found in the virgins and breeders of the C_3H mice where the extremely high Q_{O_2} values at 20 and 30 weeks respectively fall significantly in the next age group. The virgin A mice show a rise at 40 weeks which is maintained in the oldest animals as well. The temporary significant rise and fall in endogenous oxygen consumption in the male, virgin female, and breeder female C_3H mice is perhaps the most notable trend in Table I, because this constitutes a common factor independent of sex and breeding history and which is peculiar to the one strain. It should be noted that these levels of oxygen consumption are the highest in Table I.

TABLE I

OXYGEN CONSUMPTION (Q_{O_2}) OF MAMMARY TISSUE OF MALE, VIRGIN FEMALE, AND BREEDER FEMALE MICE OF THE C_{57}BL, A, AND C_3H STRAINS

Numerical values are averaged and standard errors of means are indicated. Q_{O_2} values are based on fat-free, dry weight of mammary tissue

Age in weeks	Males		Virgins		Breeders	
	Number of mice	Q_{O_2}	Number of mice	Q_{O_2}	Number of mice	Q_{O_2}
$C_{57}BL$ strain						
8 – 15	6	4.2±0.52	9	7.0±0.89	—	—
20	—	—	—	—	6	5.6±0.58
30	6	4.8±0.91	6	3.3±0.25	17	5.4±0.33
40	6	4.9±0.80	5	3.5±0.45	9	3.9±0.33
Over 50	6	4.3±0.29	6	3.7±0.38	6	3.7±0.20
A strain						
8 – 15	10	3.5±0.51	2	3.2±0.35	—	—
20	—	—	—	—	6	4.4±0.58
30	6	3.1±0.58	6	3.3±0.29	10	4.6±0.64
40	6	3.1±0.58	6	5.3±0.70	6	3.7±0.70
Over 50	6	2.7±0.40	9	4.6±0.46	5	3.7±0.85
C_3H strain						
8 – 15	5	5.1±0.30	9	4.8±0.44	—	—
20	—	—	13	6.5±0.97	8	5.1±0.78
30	6	3.0±0.58	7	4.5±0.92	6	7.3±0.58
40	6	5.6±0.83	6	3.5±0.62	16	4.9±0.42
Over 50	6	3.9±0.48	6	5.1±0.83	9	4.9±0.66

The addition of *p*-phenylenediamine to a concentration of 1.85×10^{-2} *M* in the Warburg reaction vessels resulted in a very much greater elevation of oxygen consumption than in our experiments with rats (9). The data of Table II were obtained with tissues used for the Q_{O_2} estimations reported in Table I.

There are effects in Table II which are associated with age, sex, breeding, and strain. However, it is perhaps of more interest to consider the data in Table III, which are the values of $Q_P^{O_2} - Q_{O_2}$, obtained from Tables II and I, and which represent the increase in oxygen consumption that followed the addition of *p*-phenylenediamine. According to Craig, Bassett, and Salter (1) the values in Table III are a measure of the cytochrome reserves of tissues.

It can be seen from the third table that there are considerable reserves of cytochrome in mouse mammary tissue, and on a fat-free, dry-weight basis, they constitute roughly one half the values we obtained for liver tissue of these mice, calculated on a dry-weight basis. In general, it would appear that the effect of age is to decrease the reserves of cytochrome in mammary tissue. We can offer no explanation for the few exceptions to this rule beyond

TABLE II

OXYGEN CONSUMPTION IN PRESENCE OF *p*-PHENYLENEDIAMINE ($Q_{PP}^{O_2}$) OF MAMMARY TISSUE OF MALE, VIRGIN FEMALE, AND BREEDER FEMALE MICE OF THE $C_{57}BL$, A, AND C_3H STRAINS

Numerical values are averaged and standard errors of means are indicated. $Q_{PP}^{O_2}$ values are based on fat-free, dry weight of mammary tissue

Age in weeks	Males		Virgins		Breeders	
	Number of mice	$Q_{PP}^{O_2}$	Number of mice	$Q_{PP}^{O_2}$	Number of mice	$Q_{PP}^{O_2}$
$C_{57}BL$ strain						
8 – 15	6	19.1±1.64	9	23.7±2.19	—	—
20	—	—	—	—	6	20.1±0.58
30	6	18.5±2.29	6	13.7±1.35	17	15.1±1.24
40	6	13.8±1.87	5	14.8±0.77	9	11.5±1.03
Over 50	6	17.9±1.29	6	15.6±1.05	6	12.3±0.73
A strain						
8 – 15	10	19.0±1.12	2	27.0±1.55	—	—
20	—	—	—	—	6	14.5±1.08
30	6	12.5±0.70	6	16.8±1.60	10	13.8±1.19
40	6	19.4±1.29	6	13.5±0.58	6	9.8±0.70
Over 50	6	10.3±1.58	9	12.7±0.66	5	13.9±1.47
C_3H strain						
8 – 15	5	26.2±3.68	9	18.5±0.91	—	—
20	—	—	13	16.4±2.17	8	17.9±1.32
30	6	18.4±2.58	7	14.1±1.39	6	14.7±0.91
40	6	22.4±0.83	6	17.1±1.31	16	18.1±1.15
Over 50	6	15.5±1.29	6	21.8±1.25	9	14.9±1.80

the fact that the experiments reported in this paper took approximately three years to complete in order to get all age groups represented as well as possible, and certain other imponderable factors may be responsible for these apparent deviations. It should be noted that this does not invalidate the findings with the C_3H strain, which we consider the most significant: the entire experiment with this strain was done twice, and each time we obtained high Q_{O_2} values and low cytochrome reserves as above. The data in Table III also reflect the effect of sex, breeding, and strain on cytochrome reserves but in general, it cannot be said that there are any trends as striking as the effect of age on cytochrome reserves, with the possible exception of the lowered reserves in groups of C_3H mice which are in the mammary cancer age.

The abnormally elevated endogenous oxygen consumption and the lowered reserves of cytochrome in nontumorous breast tissue of C_3H mice (Tables I and III) appeared to be the most significant of our observations. It seemed probable that these changes could be accounted for on the basis of precancerous changes since the animals were approaching or were in the age range when

TABLE III

INCREASE IN OXYGEN CONSUMPTION CAUSED BY THE ADDITION OF *p*-PHENYLENEDIAMINE TO MOUSE MAMMARY TISSUE SLICES ($Q_{P^2}^{O_2} - Q_{O_2}$)

Numerical values are averaged and standard errors of means are indicated

Age in weeks	Males		Virgins		Breeders	
	Number of mice	$Q_{P^2}^{O_2} - Q_{O_2}$	Number of mice	$Q_{P^2}^{O_2} - Q_{O_2}$	Number of mice	$Q_{P^2}^{O_2} - Q_{O_2}$
$C_{57}BL$ strain						
8 – 15	6	14.9±1.65	9	16.6±2.03	—	—
20	—	—	—	—	6	15.7±1.08
30	6	13.7±1.83	6	10.4±1.51	17	9.5±1.02
40	6	8.8±1.08	5	11.3±0.90	9	7.6±0.86
Over 50	6	13.6±1.24	6	11.8±1.03	6	8.6±0.70
A strain						
8 – 15	10	15.5±1.11	2	23.8±1.20	—	—
20	—	—	—	—	6	10.1±0.58
30	6	9.4±0.70	6	13.0±1.79	10	18.7±0.70
40	6	16.2±1.00	6	8.2±0.91	6	6.1±0.55
Over 50	6	7.6±1.50	9	8.3±0.56	5	10.2±0.98
C_3H strain						
8 – 15	5	21.03±3.00	9	13.6±0.70	—	—
20	—	—	13	10.5±0.97	8	12.3±1.07
30	6	15.4±2.00	7	9.6±0.62	6	7.1±1.00
40	6	16.8±2.29	6	13.6±2.41	16	13.2±1.00
Over 50	6	11.8±2.22	6	16.7±0.83	9	9.7±1.43

mammary tumors might be expected to develop. Consequently we determined the oxygen consumption of tumor tissue in the presence and absence of *p*-phenylenediamine, and whenever possible of breast tissue from the glands in which the tumors developed. These results are compared in Table IV with data for normal breast tissue from breeder females of the same age, 40 weeks, as recorded in the first three tables.

TABLE IV

OXYGEN CONSUMPTION (Q_{O_2}) AND RESPONSE TO *p*-PHENYLENEDIAMINE ($Q_{P^2}^{O_2}$) OF MAMMARY TUMOR TISSUE, COMPARED WITH TISSUE FROM THE GLAND CONTAINING THE TUMOR AND WITH NORMAL BREAST TISSUE, IN C_3H MICE

Numerical values are averaged and standard errors of means are indicated

Nature of tissue	Number of mice	Q_{O_2}	$Q_{P^2}^{O_2}$	$Q_{P^2}^{O_2} - Q_{O_2}$
Mammary tumor	12	9.0 ± 1.00	14.5 ± 0.97	5.5 ± 0.50
Tumor-containing breast	8	7.4 ± 0.85	17.7 ± 1.96	10.3 ± 1.32
Normal breast	16	4.9 ± 0.42	18.1 ± 1.15	13.2 ± 1.10

The data of Table IV reveal that both the tumor tissue as well as tissue from the tumor-containing breast manifest significantly higher endogenous oxygen consumption when compared with normal breast tissue from breeder mice of the same age. The reserves of cytochrome in the tumors are very much lower than normal, and this is in conformity with the findings of Craig, Bassett, and Salter (1). The reserves in the tumor-containing breast tissue are intermediate between those of tumor tissue and normal gland tissue, although the values are not significantly less than normal.

References

1. Craig, F. N., Bassett, A. M., and Salter, W. T. Cancer Research, 1 : 869. 1941.
2. Greenstein, J. P. Biochemistry of cancer. Academic Press Inc., New York. 1947.
3. Rawlinson, H. E. and Pierce, G. B. Endocrinology, 46 : 426. 1950.
4. Rawlinson, H. E. and Pierce, G. B. Proc. Am. Assoc. Cancer Research, Cancer Research, 12 : 289. 1952.
5. Richardson, F. L. Anat. Record, V, 111 : 669. 1951.
6. Tuba, J. Cancer Research, 12 : 113. 1952.
7. Tuba J. Can. J. Med. Sci. 31 : 41. 1953.
8. Tuba J. and Fraser, M. S. Can. J. Med. Sci. 30 : 14. 1952.
9. Tuba, J., Rawlinson, H. E., and Shaw, L. G. Can. J. Research, E, 28 : 217. 1950.

STATISTICAL METHODS IN MEDICAL RESEARCH

II. SAMPLE SIZES REQUIRED IN EXPERIMENTS INVOLVING ALL-OR-NONE RESPONSES[1]

By Donald Mainland[2] and Marion I. Sutcliffe[3]

Abstract

In order to obtain a reliable estimate of the sample size (number of individuals) required in an experiment, an investigator must first specify: (1) what risk he is willing to run of mistakenly concluding that his different experimental treatments have produced different effects; (2) what the magnitude of the effect produced by one of the treatments is likely to be; (3) what magnitude of difference between treatment effects would be important to him; (4) what risk he is willing to run of failing to detect a difference when it is of that magnitude—the risk of an unsuccessful experiment. Difficulties in answering these questions are discussed with reference to five examples from different types of medical research. For experiments involving the comparison of two treatments that produce an all-or-none effect such as death or survival, a table derived from binomial expansions is presented, which, for samples containing up to 100 individuals, shows the probabilities of successful experiments for different magnitudes of treatment effects.

Introduction

In the planning of any investigation an essential question is: How much evidence is needed? In human and animal experimentation this commonly implies: How many individuals are required in the samples? Reliable estimates of sample size are, if anything, more important in research on human beings than in animal research, in order to reduce both the number of inconclusive investigations and the amount of needless experimentation. The answer to the question regarding required sample size is, however, not simple, for it depends on the meaning of "required", which is apparently not clear to many investigators who bring the question to a statistical laboratory.

Some clarification is achieved by noting the two kinds of error to which all judgments regarding the identity or difference between two things or phenomena are liable. One can err by judging that A and B are different when they are really alike, or by judging that they are alike when they are really different. As applied to statistical tests of significance, e.g., in the comparison of two sample means or two percentage frequencies, the errors are defined thus:

An error of the first kind or *Type I error* occurs if a difference is proclaimed "significant" when there is no "real" difference, i.e., when the samples actually belong to the same population.

An error of the second kind or *Type II error* occurs if a difference is proclaimed "not significant" when there is a "real" difference, i.e., when the samples actually belong to different populations.

[1] *Manuscript received June 8, 1953.*
Contribution from the Department of Medical Statistics, New York University College of Medicine, with the aid of grants from the Abbott Laboratories; the Milbank Memorial Fund; Parke Davis and Company; Charles Pfizer and Company; and the Squibb Institute for Medical Research.

[2] *Professor of Medical Statistics.*
[3] *Statistician.*

In deciding what risks he is willing to run, the investigator should consider, for each particular problem, what would be the respective consequences of a Type I error and a Type II error. These consequences may include such actions as futile further experimentation, unwarranted change of clinical treatment, unjustifiable adherence to the less efficacious of two treatments, erection of a hypothesis on a false foundation, and, very frequently, waste of time and money.

Four Preliminary Decisions

For ease of discussion we shall suppose that an investigator intends to apply two treatments, *V* and *W*, in order to determine whether their effects, assessed either by measurement (e.g., blood sugar level) or by frequencies (e.g., percentage mortality) are different. To make his requirements regarding sample size specific, he has to answer the following four questions:

1. If there is actually no difference between the effects of *V* and *W*, what risk are you willing to run of mistakenly concluding that there is a difference? That is, what risk of a Type I error will you accept?

2. What do you think is a likely population value for either the *V*-treated or the *W*-treated group?

3. If there is a real difference between the effects of *V* and *W*, what size of difference is important to you?

4. If there is a real difference of the size specified in answer to Question 3, what risk are you willing to run of failing to detect a difference? That is, what risk of a Type II error will you accept?

Each of these questions requires amplification.

1. Erroneous Inference of a Difference—Type I Error

It is customary for biological and medical workers to set the maximum risk of a Type I error at 5%, and this is the basis of the ordinary tests of significance. When the result of a test (such as Chi-square or the *t* test) shows a probability (*P*) of chance occurrence less than 0.05, it implies that, if chance alone were operating, fewer than 5% of similar experiments would produce quantities (Chi-square, *t*, etc.) as great as, or greater than, the one obtained from the actual experiment. Adopting the 5% level through a lifetime of research, therefore, the investigator ensures that, of all experiments where chance alone is responsible for observed differences, not more than 5% will lead him into error.

2. Estimated Population Values

By a "population" value is meant the value that would be approached more and more closely by taking larger and larger *random* samples of the subjects, material, or phenomena under investigation. Various methods of obtaining estimates of these values will be discussed later.

3. Important Size of Population Difference

If, in answer to Question 3, an investigator said that any real difference, however small, would be important, he would commit himself to ever larger samples; and therefore what is necessary is a statement such as the following: "If with treatment *V* the population percentage mortality would be 30 and with treatment *W* it would be 40, I wish to have considerable confidence that my experiment will reveal a superiority of the *V* treatment by showing a difference in mortality in my samples that will be significant at the 5% level." A similar statement could be phrased in terms of measured values, e.g., a reduction of blood sugar by so many mgm. per 100 ml. would correspond to the mortality difference.

4. Failure to Detect a Population Difference—Type II Error

An experiment that shows a significant difference in the same direction as the population difference (e.g., a lower mortality with *V* than with *W*) can be called a *successful* experiment, and one that does not show such a significant difference is unsuccessful. The percentage of Type II errors, then, is the percentage of unsuccessful experiments that would occur in a long series of experiments conducted under certain specified conditions regarding population differences. Obviously, for any particular population difference, the larger the sample the more likely will it be to reflect that difference and to provide a verdict of significance when tested. Therefore the risk of a Type II error can be set at 1%, 5%, or higher, depending on the size of samples that the investigator is able and willing to take. Instead of specifying the risk of error, the investigator can specify his confidence in a successful experiment; e.g., a 5% error corresponds to 95% successful experiments.

It should be noted, of course, that "success" does not imply that the difference observed in the experiment will be numerically the same as the population difference. It may be higher or lower than that, but it will be large enough to be statistically significant.

Commonly used simple methods of estimating sample size mostly ignore the foregoing questions, by assuming that the difference between larger samples, and the variation between individuals in the samples, will remain the same, as shown by smaller samples already observed by the investigator or someone else. The estimates so derived are useful if they reveal that, with the material and facilities available, a large enough sample could not be obtained; but the assumptions are such that the chances of a successful experiment are only, roughly speaking, about 50 : 50. When the four questions have been answered much more satisfactory estimates can be made. If the data comprise measurements the required sample sizes can be estimated by methods (1) that are not difficult for anyone who is familiar with "Student's" *t* and the variance ratio (Snedecor's *F*). If, however, the data are classified by attributes, *A* and *not-A*, i.e., if they record "all-or-none" responses such as deaths and survivals, the calculation is much more complicated and time-consuming. Therefore we have prepared the accompanying table (Table I)

TABLE I

PERCENTAGES OF SUCCESSFUL EXPERIMENTS (% S) IN RELATION TO SAMPLE SIZE (N) AND TO PERCENTAGES OF *A*'S IN POPULATIONS *V* AND *W*

N	% S
V:1%, W:5%	
5	0.0
10	0.0
15	0.0
20	0.2
30	0.3
50	2.4
70	7.6
100	19.4
V:1%,W:10%	
5	0.0
10	0.1
15	1.1
20	3.6
30	5.6
50	27.2
70	51.5
100	76.9
V:1%,W:15%	
5	0.2
10	0.9
15	5.4
20	14.3
30	23.1
50	63.7
70	86.1
100	97.3
V:1%,W:25%	
5	1.5
10	7.1
15	27.7
20	51.5
30	71.0
50	96.5
70	99.7
100	100.0
V:1%,W:33%	
5	4.2
10	18.8
15	52.9
20	77.5
30	92.0
50	99.8
70	100.0
V:1%,W:50%	
5	18.0
10	57.9
15	90.3
20	98.1
30	99.9
50	100.0
V:1%,W:67%	
5	45.1
10	89.1
15	99.3
20	100.0
V:1%,W:75%	
5	61.3
10	96.0
15	99.9
20	100.0
V:1%,W:85%	
5	81.6
10	99.3
15	100.0
V:1%,W:90%	
5	90.2
10	99.8
15	100.0
V:1%,W:95%	
5	96.7
10	100.0
V:1%,W:99%	
5	99.6
10	100.0
V:5%,W:10%	
5	0.0
10	0.1
15	0.6
20	1.7
30	1.9
50	6.3
70	11.4
100	19.0
V:5%,W:15%	
5	0.2
10	0.6
15	3.0
20	7.0
30	9.3
50	24.3
70	40.7
100	58.6
V:5%,W:25%	
5	1.2
10	4.8
15	16.6
20	29.9
30	43.6
V:5%, W:25% —*Cont.*	
50	74.8
70	90.8
100	98.1
V:5%,W:33%	
5	3.5
10	13.0
15	34.7
20	53.3
30	73.1
50	94.8
70	99.2
100	100.0
V:5%,W:50%	
5	15.1
10	43.1
15	73.6
20	89.4
30	98.2
50	100.0
V:5%,W:67%	
5	38.9
10	75.8
15	94.8
20	99.3
30	100.0
V:5%,W:75%	
5	53.8
10	87.3
15	98.4
20	99.9
30	100.0
V:5%,W:85%	
5	73.7
10	96.4
15	99.9
20	100.0
V:5%,W:90%	
5	83.1
10	98.7
15	100.0
V:5%,W:95%	
5	91.4
10	99.7
15	100.0
V:5%,W:99%	
5	96.7
10	100.0
V:10%,W:15%	
5	0.1
10	0.4
15	1.4
20	2.7
30	2.9
50	5.9
70	9.6
100	13.8
V:10%,W:25%	
5	1.0
10	2.9
15	8.5
20	14.4
30	21.3
50	41.0
70	58.5
100	76.2
V:10%,W:33%	
5	2.7
10	8.0
15	19.6
20	31.1
30	47.6
50	75.7
70	90.1
100	97.6
V:10%,W:50%	
5	12.1
10	29.5
15	53.6
20	74.2
30	91.4
50	99.4
70	100.0
V:10%,W:67%	
5	32.0
10	60.9
15	85.5
20	96.5
30	99.7
50	100.0
V:10%,W:75%	
5	45.1
10	75.9
15	94.4
20	99.2
30	100.0
V:10%,W:85%	
5	63.9
10	90.9
15	99.1
20	99.9
30	100.0
V:10%,W:90%	
5	73.6
10	95.8
15	99.8
20	100.0
V:10%,W:95%	
5	83.1
10	98.7
15	100.0
V:10%,W:99%	
5	90.2
10	99.8
15	100.0
V:15%,W:25%	
5	0.7
10	1.7
15	4.2
20	6.6
30	9.2
50	17.1
70	25.3
100	35.9
V:15%,W:33%	
5	2.1
10	4.8
15	10.6
20	17.2
30	27.1
50	48.2
70	65.4
100	81.6
V:15%,W:50%	
5	9.5
10	19.9
15	37.1
20	57.3
30	77.7
50	95.8
70	99.4
100	100.0
V:15%,W:67%	
5	26.0
10	48.0

TABLE I—*Concluded*

PERCENTAGES OF SUCCESSFUL EXPERIMENTS (% S) IN RELATION TO SAMPLE SIZE (N) AND TO PERCENTAGES OF *A*'S IN POPULATIONS *V* AND *W*—*Concluded*

N	% S	N	% S	N	% S	N	% S	N	% S
V:15%, W:67% —*Cont.*		*V:15%, W:99%*		*V:25%, W:75%*		*V:25%, W:99%*		*V:33%, W:75%* —*Cont.*	
15	73.8	5	81.6	5	24.4	5	61.3	70	99.9
20	90.0	10	99.3	10	42.1	10	96.0	100	100.0
30	98.4	15	100.0	15	67.6	15	99.9		
50	100.0			20	83.4	20	100.0	*V:33%, W:85%*	
		V:25%, W:33%		30	97.0			5	26.0
V:15%, W:75%		5	1.2	50	99.9	*V:33%, W:50%*		10	48.0
5	37.4	10	1.6	70	100.0	5	3.6	15	73.8
10	64.2	15	2.8			10	4.1	20	90.0
15	87.7	20	4.5	*V:25%, W:85%*		15	7.1	30	98.4
20	96.6	30	6.1	5	37.4	20	11.6	50	100.0
30	99.8	50	10.0	10	64.2	30	18.7		
50	100.0	70	14.0	15	87.7	50	34.3	*V:33%, W:90%*	
		100	19.3	20	96.6	70	46.4	5	32.0
V:15%, W:85%		*V:25%, W:50%*		30	99.8	100	64.4	10	60.9
5	54.4	5	5.7	50	100.0			15	85.5
10	83.3	10	8.6			*V:33%, W:67%*		20	96.5
15	97.2	15	15.7	*V:25%, W:90%*		5	10.8	30	99.7
20	99.6	20	26.9	5	45.1	10	16.4	50	100.0
30	100.0	30	41.3	10	75.9	15	30.1		
		50	67.5	15	94.4	20	43.8	*V:33%, W:95%*	
V:15%, W:90%		70	83.1	20	99.2	30	68.4	5	38.9
5	63.9	100	94.5	30	100.0	50	91.5	10	75.8
10	90.9					70	97.8	15	94.8
15	99.1	*V:25%, W:67%*		*V:25%, W:95%*		100	99.8	20	99.3
20	99.9	5	16.4	5	53.8			30	100.0
30	100.0	10	27.6	10	87.3	*V:33%, W:75%*			
		15	48.3	15	98.4	5	16.4	*V:33%, W:99%*	
V:15%, W:95%		20	66.1	20	99.9	10	27.6	5	45.1
5	73.7	30	87.9	30	100.0	15	48.3	10	89.1
10	96.4	50	98.8			20	66.1	15	99.3
15	99.9	70	99.9			30	87.9	20	100.0
20	100.0	100	100.0			50	98.8		

which, although limited in scope by the amount of available computing aid, covers a fair range of the sample sizes used in small-scale clinical and laboratory research—up to samples containing 100 individuals. The population values above 15% correspond to the round-figure estimates that are often the closest approximation possible—1/4, 1/3, 1/2, 2/3, and 3/4.

The table is applicable to investigations in which the 5% level of significance is to be adopted when the samples are tested at the end of the experiment. It is restricted to equal samples because these have two advantages:

1. For any particular total number of individuals, they provide more information than do unequal samples (for illustrations, see (7)).

2. The significance test can be applied by the use of tables that entail little or no calculation (9, 10).

Method of Using Table I

For simplicity in the first example, deaths in an animal experiment will be used. The experiment is designed to compare the fatality rates (percentage of deaths) of animals under two treatments. From some information obtained in other experiments an investigator believes that the fatality under one treatment would in the long run be about 75%. This is the assumed population percentage of *A*'s for that treatment, and the investigator states that if the population percentage of deaths under the other treatment is as low as 33% he wishes to make N, the number of animals in each of the two samples, large enough to ensure a 90% confidence that the experiment will be successful, i.e., large enough to ensure that 90% of experiments conducted under these conditions would show significant differences in the same direction as in the populations.

To enter Table I, we call the lower of the two percentages (33%) the value for the *V* population and the higher (75%) the value for the *W* population. By examining this part of the table (*V : 33%, W : 75%*) we see that with samples of 30 the percentage of successful experiments would be 87.9, and with samples of 50 animals 98.8% of the experiments would be successful. Samples of 40 would therefore ensure more than 90% success under the specified conditions. (The same estimate would be reached, of course, by entering the table with the percentages of survivors—*V : 25%, W : 67%*.)

Inspection of other parts of the table reveals two important features:

1. If an investigator desires the same assurance (90% probability of success) when the *V* population is the same as before (33%) but the *W* percentage is closer to it (67 instead of 75%) the samples required are larger, approximately 50 individuals in each; and at *V : 33%, W : 50%* samples of 100 give only 64.4% successful experiments.

2. The mere difference between the population percentages is not a satisfactory guide to sample sizes. Thus, for *V : 1%, W : 15%* (a difference of 14%) two samples of 70 provide 86.1% successful experiments, whereas for *V : 10%, W : 25%* (a difference of 15%) samples of 70 give only 58.5% probability of success.

Difficulties in Preliminary Decisions

The difficulty in estimating sample size is not in using the table but in answering the second and third of the four questions necessary in the use of the table. Estimates of population percentages, sometimes very crude, sometimes fairly precise, can often be made from previous observations by the investigator or by others. In all such estimates, too great trust in the previous observations should be avoided by finding their confidence limits, which is easily done from tables (2, 3, 9). Thus, if 5 subjects in a sample of 50 showed a certain reaction, *A*, the 95% confidence band for the percentage in the population, randomly represented by the sample, reaches from 3.3 to

21.8% *A*'s. Therefore, on the customary standard, it would not be unreasonable for the investigator to assume a population percentage anywhere between these limits, and he might wish to try several different values in finding required sample sizes.

In practice, as the Examples will show, the four preliminary questions are very often most appropriately considered in a different sequence from the one in which they have been enumerated above.

Sometimes an investigator asks a statistician what size of difference should be considered important and what is the desirable probability of a successful experiment. All that the statistician can do is indicate the kinds of factors that should be taken into account. Sometimes failure can be expressed in man-hours or dollars, but often features that are difficult to measure have to be considered, such as the educational benefit of even an "unsuccessful" experiment to a young research worker, or the possibility that an experienced investigator, working on a certain problem, may find a clue to something of far greater importance than what he is seeking.

Inferences After the Experiment

Whatever estimates are made at the outset, the validity of any inference from the experiment can be assured only by the proper design and performance of the experiment itself, including allocation of treatments by an automatic process of randomization, such as the use of random numbers—an essential part of a sound experiment, but still too frequently omitted (3 – 5).

Examples

Five examples, modeled on problems to which Table I has been applied, illustrate some of the points to be considered in choosing sample size.

Example 1. Clinical Trial of a Sedative

In the trial of a new drug for immediate postoperative sedation, because of the shortness of time during which effects were to be observed, it was impossible to administer placebo and drug to the same patient on different occasions, and therefore some patients were to receive placebo alone and others the drug alone. The physician who was undertaking the trial stated that he would expect about 25% of patients, of the type concerned, to register benefit from a placebo, and that he would not consider the new drug worth further trial unless it would benefit another 25% of patients. In Table I, under *V : 25%*, *W : 50%*, it is found that 100 patients on placebo and 100 on the drug would give 94.5% probability of a successful experiment if the population percentages were as stated.

Example 2. Immunization against a Carcinogen

When a carcinogen was painted on the skin of 10 mice all developed tumors, but when it was painted on the skin of 10 mice that had previously been treated with a possible tumor-inhibiting substance, only seven developed tumors during the same period. The difference is not significant at the 5% level; indeed as many as 21% of experiments would show differences of this magnitude or larger, even if nothing but chance were responsible for the differences (2, 9).

Although it is possible that no mice of this strain, treated as were the controls (without potential inhibitor) would fail to develop tumors, one cannot, from the sample itself, have confidence that fewer than 30.9% would be tumor-free (upper binomial confidence limit (95%) for zero *A*'s in a sample of 10). However, from other experience with fairly similar carcinogen treatment the investigator believed that 10% tumor-free would be a much more likely upper limit.

With $V = 10\%$, therefore, W will be the population of mice treated by the potential inhibitor, and if W had 33% tumor-free mice, 70 animals in each of the two samples would be necessary to give a 90.1% probability of detecting the tumor-inhibiting effect. A population difference of 5% ($V : 10\%$, $W : 15\%$), although a very important difference in cancer research, would relatively seldom produce a significant difference in tumor incidence, even with samples of 100 mice (13.8% of experiments successful).

Example 3. Antibody Development and Age at Immunization

Of seven children who were actively immunized against diphtheria at birth, only two had detectable antibody titer (greater than 0.002 units) one month afterward. Of six who were immunized three months after birth, five had detectable titers one month later. This is an example of observations in which measurements are made but are not easy to use in the analysis (6), because the lowest (and often the highest) titers are not definite values. The difference in the proportions (two out of seven and five out of six) is not significant, but the pediatrician was disinclined to stop the research if the real difference were as great as suggested by the samples (29% versus 83%) and if another dozen babies on each treatment, added to those previously studied, would then show a significant difference.

With samples of six and seven, the observed percentages may, as confidence limits show, be far from the population values, but an estimate of sample size can be given as suggested by taking W as 85%, and V at two levels, 25% and 33% (to enclose the observed 29%). With two samples of 20 the probability of success would, on the specified assumption, be at least 90%.

Example 4. Association Between Blood Groups and Disease

It was proposed to seek for a relationship between a certain disease and blood groups by making a comparison between the frequency of the different groups in patients and the frequency in healthy persons from the population served by the same hospital. From any nonexperimental or "survey" investigation the danger of bias can never be wholly removed. Therefore untestable assumptions should be stated before the investigation begins and again in the report (8); and it should be realized that any conclusions are open to several interpretations, some of which may be unsuspected.

If, although fully aware of the danger, the investigator decides to conduct the survey, he can often use some published data as an indication of population values. In the blood group problem the figure 14% in Group B for whites

was taken, and it was suggested that the possible percentage of this blood group in the patients might be 25%. The investigator said that, on these assumptions, he would be willing to take a 50 : 50 chance of showing a significant difference in the frequency of Group B between the patients and the healthy subjects. Table I, at *V : 15%, W : 25%*, shows that even this low degree of confidence cannot be given by samples of 100. Rough linear extrapolation indicated that samples of 150 would be required.

The willingness to take such low odds of success often seems to arise from failure to appreciate what a 50 : 50 chance of success really implies—that even when the things that an investigator is seeking are really present he is willing to risk failing to find them in 50% of his investigations.

Example 5. Effect of Oxygen Concentrations on Premature Infants

A certain disorder in premature infants was suspected to be due, in part at least, to the high concentration of oxygen customarily administered in the incubators. A pediatrician proposed to administer the usual high concentration to one group of infants but to give to another group, of equal size, a lower concentration, the two treatments being allocated to individual infants strictly at random. He stated that within the 12 months which he proposed to spend on the investigation he could expect only 60 infants (30 for each group) in the low weight range where the disorder most commonly occurs; and he asked what population difference in the incidence of the disorder would be sufficient to ensure an 80% probability of a successful experiment.

Information on the incidence of the disorder was meager, and therefore a number of different population values were examined, N being taken as 30 throughout. On the assumption that the reduction of oxygen would lower the incidence of the disorder, *V* represented the low-oxygen population, *W* the high-oxygen population.

Under *V : 1%*, the values that enclose the desired 80% successes are:

W : 25%, percentage of successes = 71.0.
W : 33%, percentage of successes = 92.0.

Although there is not yet sufficient material to show the precision of linear interpolation between population percentages, this method can be accepted as giving the rough approximation that is usually desired in such estimates. The difference between 71.0 and 92.0 is 21.0; the difference between 71.0 and the required 80.0 is 9.0; and the difference between 25% and 33% is 8%. We therefore estimate the *W* population percentage as $25 + (9/21) \times 8 = 25 + 3.4 = 28.4\%$. The difference between *V* and *W* is therefore 27.4, and may be given as something between 25% and 30%.

Similarly, with $V = 5\%$, linear interpolation gives $W = 37.7\%$ as the value required for 80% confidence in a successful experiment with 30 children in each sample. The difference between *V* and *W* must therefore be taken as at least 30%.

Production of Table I

Basis of the Table

The basis on which Table I was constructed can be seen by examining first the relative frequencies of all possible random samples of five from populations *V : 10%* and *W : 67%*. These are given by the binomial expansions $(0.1 + 0.9)^5$ and $(0.67 + 0.33)^5$ as follows:

V = 10%			*W* = 67%		
Sample composition		Relative frequency	Sample composition		Relative frequency
A's	*Not-A*'s		*A*'s	*Not-A*'s	
0	5	0.5905	0	5	0.0039
1	4	0.3280	1	4	0.0397
2	3	0.0729	2	3	0.1613
3	2	0.0081	3	2	0.3275
4	1	0.0005	4	1	0.3325
5	0	0.0000	5	0	0.1350
		1.0000			0.9999

If a sufficiently large number of random samples were taken, one from *V* and one from *W*, all possible combinations would be met, and the proportions would be found by multiplying together the respective frequencies shown above. Thus, the frequency of *V*(0 *A*'s, 5 *not-A*'s) along with *W*(2 *A*'s, 3 *not-A*'s) would be $0.5905 \times 0.1613 = 0.0952$; i.e., 9.52% of all pairs of random samples would have this composition.

If in an actual experiment, with population values of course unknown, this sample were found, the difference between 0,5 and 2,3 would be tested by Chi-square, or a more accurate verdict regarding its significance would be found directly from tables (9,10), and the difference would be pronounced not significant at the 5% level. Therefore, although the investigator would be unaware of it, the experiment would have been unsuccessful, in that it had failed to provide significant evidence that the populations differed.

Relative frequencies less than 0.0000 are negligible. Therefore, out of all the possible pairs of samples from these populations only three types would provide significant differences, namely: *V*(zero *A*'s) with *W*(5 *A*'s); *V*(zero *A*'s) with *W*(4 *A*'s); and *V*(1 *A*) with *W*(5 *A*'s). The combined frequencies of all these, found by multiplication as in the above example, is 32.0% of the total possible combinations, and this value is recorded in Table I at *V: 10%, W:67%*.

Misleading Experiments

Even when the *W* population percentage (e.g., fatality) is greater than the *V* population percentage, pairs of samples often show the reverse difference, *V* sample frequency greater than *W* sample frequency, and sometimes the difference is significant. The conclusion from such an experiment would be that treatment *V* was more lethal than treatment *W*, whereas the reverse is true. In the area covered by Table I this would occur only occasionally, and the proportion of such misleading experiments, for any particular pair of population percentages and sample size, would not be more than 0.2% (1 in 500). It is most likely to happen when population percentages are relatively close together, and the locations are as follows:

V: 10%, W: 15% (and *V: 85%, W: 90%*), with N = 15 to 100
V: 15%, W: 25% (and *V: 75%, W: 85%*), with N = 5 to 30
V: 25%, W: 33% (and *V: 67%, W: 75%*), with N = 5 to 70
V: 25%, W: 50% (and *V: 50%, W: 75%*), with N = 5
V: 33%, W: 50% (and *V: 50%, W: 67%*), with N = 5

Computation

The above example shows the outline of the procedure, including assessment of significant differences by exact tables. All the binomial expansions were carried to seven decimal places. The expansions for sample sizes 5 to 30 were obtained from the Bureau of Standards tables (11); the others were computed in this laboratory, and each expansion was found to total to within ± 0.00004 of unity. The terms were then rounded to four places for further calculation. Each stage of the computation was thoroughly checked, and as an extra safeguard each block of figures in Table I was computed in two forms independently. Thus, the block headed "*V: 25%, W: 33%*" was calculated also in the equivalent form "*V: 67%, W: 75%*". The percentage of successful experiments as reported in Table I can be taken as correct to the first decimal place, i.e., its error will rarely be as great as ± 0.1.

Enlargement of Table I

It is hoped that Table I may be enlarged, but because the process is expensive it is desirable to concentrate effort in directions of greatest value to research workers. Readers who use the table could assist greatly by indicating their opinions regarding the relative usefulness of the following features: (1) values of N above 100; (2) introduction of the 1% level of significance; (3) smaller intervals between values of N below 100; (4) narrower intervals between population percentages; (5) population values below 1%. Suggestions of this kind, and any other comments, sent to this Department at 477 First Avenue, New York, 16, N.Y., would be much appreciated.

Acknowledgments

The authors wish to thank Mrs. Joan M. Hansen, Mrs. Catherine Mescal, and Miss Ada Kimmelman for the major part of the computation, and Mrs. Lee Herrera for constructive criticism of the report.

References

1. HARRIS, M., HORVITZ, D. G., and MOOD, A. M. J. Am. Stat. Assoc. 43 : 391. 1948.
2. MAINLAND, D. Can. J. Research, E, 26 : 1. 1948.
3. MAINLAND, D. Elementary medical statistics. The principles of quantitative medicine. W. B. Saunders Co., Philadelphia. 1952.
4. MAINLAND, D. Chance and random sampling. Methods in medical research, 6. The Year Book Publishers, Chicago. In press.
5. MAINLAND, D. The modern method of clinical trial. Methods in medical research, 6. The Year Book Publishers, Chicago. In press.
6. MAINLAND, D. Nonmetrical tests of measurement data. Methods in medical research, 6. The Year Book Publishers, Chicago. In press.
7. MAINLAND, D. and HERRERA, L. Sample sizes. Methods in medical research, 6. The Year Book Publishers, Chicago. In press.
8. MAINLAND, D. and HERRERA, L. Clinical surveys. Methods in medical research, 6. The Year Book Publishers, Chicago. In press.
9. MAINLAND, D., HERRERA, L., and SUTCLIFFE, M. I. Statistical tables for use with binomial samples. In preparation.
10. MAINLAND, D. and MURRAY, I. M. Science, 116 : 591. 1952.
11. NATIONAL BUREAU OF STANDARDS. Tables of the binomial probability distribution. Applied Math. Ser. 6. U.S. Govt. Printing Office, Washington. 1950.

THE PREVENTION OF LIVER NECROSIS BY ALPHA-TOCOPHEROL, METHIONINE, AND CYSTEINE BY ORAL AND PARENTERAL ROUTES[1]

By J. R. McLean[2] and J. M. R. Beveridge

Abstract

Groups of 10 male weanling rats of the Sprague-Dawley strain were fed a basal necrogenic diet supplemented with alpha-tocopherol, DL-methionine, and L-cysteine. The amount of supplement contained in the daily ration was administered parenterally to similar groups of rats. All three antinecrogenic agents employed exerted some action when given either in the diet or by injection. Alpha-tocopherol completely prevented the development of liver necrosis by both routes. On an equisulphur basis, methionine was obviously more effective than cysteine by either method of administration. The experiment threw no light on the question of the site of action of these compounds. The low antinecrogenic potency of cysteine compared to methionine or to cystine has posed a problem yet to be explained.

Introduction

A number of investigators have thoroughly substantiated the fact that when young rats are fed certain diets low in the sulphur-containing amino acids, methionine and cystine, and in alpha-tocopherol, they succumb within a few weeks owing to acute hepatic damage (8, 9, 5, 13, 18, 19, 14, 1, 2). However, the precise sequence of events leading to the development of the liver lesion has not yet been elucidated. Gyorgy and his collaborators (11) have suggested that the intestinal flora may be responsible for the production of a substance "with which the liver, in the absence of vitamin E or of the sulphur-containing amino acids as 'detoxifying agents', is unable to cope". This suggestion offers a reasonable explanation for the antinecrogenic properties of bacteriostatic agents such as aureomycin (2, 11, 12). Further weight is given to the hypothesis that the intestinal flora may be involved in the production of the lesion by the fact that the left half of the liver is usually more severely involved than the right half and, as has been demonstrated by Copher and Dick (4) and Glynn and Himsworth (7), the portal blood from the left half of the large intestine, a segment with a relatively high bacterial count, drains into the left half of the liver. Glynn and Himsworth, however, believe that the predilection for the lesion to be more severe in the left half of the liver may be explained by the striation of portal blood flow causing most of the protective nutrients absorbed from the small intestine to be channeled into the right half of the liver.

Additional experimental results have been obtained recently by both Gyorgy, Stokes, and Goldblatt (10) and ourselves (17), which re-emphasize the possible etiological role of the intestinal flora in the process under discussion. The former workers found that when aureomycin was used in successive experi-

[1] *Manuscript received June 26, 1953.*
Contribution from the Department of Biochemistry, Queen's University, Kingston, Ont. A preliminary report of this investigation was given before the Canadian Physiological Society at Montreal, Que., October 1952.

[2] *The work reported in this paper was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy by J. R. McLean.*

ments in their laboratory over a period of one and a half years it gradually lost its antinecrogenic property. Furthermore when experiments were set up with identical conditions in two other laboratories where such work had never been conducted previously the pronounced antinecrogenic effect originally found by Gyorgy *et al.* was again observed. This experience led the investigators to postulate "that in the course of 1 and 1/2 years either aureomycin resistant strains of intestinal bacteria developed in the animal room and were transmitted from one experiment to the following one or that the virulence of some unrecognized infectious factor, such as a virus, increased to such an extent that the aureomycin effect was obscured" (10).

A somewhat similar sequence of events was noted recently in our own laboratory. We had previously reported (16), as had also Dam and Granados (6), that the elimination of fat from a basal necrogenic ration prevented the development of liver necrosis. In the course of subsequent studies to determine which part of the dietary fat was responsible for potentiating the necrogenic effect of the basal diet, lard was fractionated and as each preparation was obtained it was tested along with a diet free from fat as a control. In successive experiments, the latter diet led to an increasing incidence of liver necrosis. It is possible that the initial feeding of the fat-free diet might have altered the intestinal flora in such a way that little or no necrogenic toxin was produced. With subsequent feedings of this diet the organisms responsible for the production of the toxin might have become adapted to the altered dietary regime and were again able to produce the hepatotoxin in lethal amounts.

The wide dissimilarity in molecular structure of alpha-tocopherol and the sulphur-containing amino acids, methionine and cystine, and the fact that these compounds exert a similar prophylactic action in the prevention of acute liver necrosis has led to much speculation on the relationship between them and on their mode of action. It is possible that this situation might be explained on the basis of these compounds being required at different points in a particular metabolic process. The possibility also exists that one of these substances might act exclusively within the intestinal tract and another in some part of the body after absorption. Such a circumstance would serve to explain the similar prophylactic action of these structurally divergent substances.

The feasibility of testing this thesis by the administration of these compounds by mouth and by injection seemed reasonable. Furthermore, such an experiment might throw light on the intestinal toxin theory to which reference has just been made.

Experimental

Seventy male rats of the Sprague-Dawley strain were divided into groups of 10, with an average initial weight of about 55 gm., and were offered eight gm. of the test diets per day. The composition of the basal diet was as follows: primary grown yeast (*Saccharomyces cerevisiae*), 18%; lard, 5%; sucrose, 68.98%; salt mixture, 3%; vitamin mixture, 1% (16); cod liver oil, 2%; celluflour, 2%; desiccated thyroid, 0.02%. The basal diet was fed alone and with

supplements of the sulphur-containing amino acids and alpha-tocopherol at levels indicated in Table I. The amount of supplement contained in the daily ration was administered by injection each day to other groups. Since the insolubility of cystine precluded its administration by injection, cysteine was used in its place. The solubility of methionine limited the amount that could be given parenterally but no suitable substitute was available. The amino acid solutions were freshly prepared every morning, the compounds being dissolved in water. In the case of cysteine hydrochloride the preparation was adjusted to pH 7.4. The experiment was terminated after a period of 105 days. Although in practically all cases the state of the liver could be determined by gross inspection, the final judgment was based upon an examination of histological sections prepared from the three main lobes of the liver and stained with haematoxylin and eosin.

TABLE I

ALPHA-TOCOPHEROL, METHIONINE, AND CYSTEINE BY ORAL AND PARENTERAL ROUTES

Daily supplement	Rats dying of necrosis	Survival time		Total incidence of necrosis
		Range	Av.	
None	10	23 – 70	(39)	10/10
4.5 mgm. alpha-tocopherol orally	0	—		0/10
4.5 mgm. alpha-tocopherol parenterally	0	—		0/10
16 mgm. DL-methionine orally	4	63 – 73	(70)	4/10
16 mgm. DL-methionine parenterally	7	42 – 82	(61)	7/10
40.3 mgm. L-cysteine orally	7	45 – 73	(61)	7/10
40.3 mgm. L-cysteine parenterally	8	49 – 82	(71)	8/8*

** Two animals died at the beginning of the experiment and are not included in the incidence of liver necrosis.*

Results and Discussion

The results obtained are shown in Table I. Ten out of 10 animals fed the basal diet died of liver necrosis after an average interval of 39 days. Since the incidence of necrosis was zero with alpha-tocopherol given orally and parenterally, it is probable that this antinecrogenic agent does not act exclusively in the digestive tract. However, one must keep in mind the fact that certain compounds when administered parenterally can enter the lumen of the gastrointestinal tract. Since the level of alpha-tocopherol used here was sufficiently high to prevent necrosis by both oral and parenteral routes no conclusions can be drawn as to the comparative efficacy of the two routes of administration.

A comparison of the two groups receiving methionine indicates that this substance exerts some action when given by injection but may be more effective in the diet. The difference could not be shown to be statistically significant.

Cysteine shows a similar pattern exerting some action by injection but perhaps slightly more when given by mouth. Again this difference could not be shown to be statistically significant. However, the small effect of cysteine by either route is surprising since previous work has shown that 0.5% cystine completely prevents liver necrosis (15). It may also be noted that cysteine has a much less marked effect than methionine on a mole : mole basis.

After this experiment was completed, Schwarz described somewhat similar results on the relative effects of dietary cysteine and cystine in a paper delivered at the 1952 meeting of the Federated Societies (20). However, he found that methionine and cysteine had essentially the same antinecrogenic potency whereas we have found that although three times as much amino-acid-sulphur was supplied by the cysteine, the methionine supplement given by mouth or parenterally was possibly more effective in preventing acute massive liver necrosis.

When one considers that the antinecrogenic effect of methionine is thought to be due to its conversion to cystine in the body it becomes difficult to explain the relatively poor antinecrogenic action of cysteine compared to methionine since the latter amino acid, in order to give rise to cystine, must presumably first of all form cysteine (3). This result raises the interesting possibility that methionine may not need to be converted into cystine in order to exert its prophylactic action.

Since both Schwarz (20) and ourselves find that cysteine is much less effective in preventing liver necrosis than is cystine, one is led to the conclusion that under the dietary conditions used the conversion of cysteine to cystine is impaired.

Acknowledgments

The authors wish to acknowledge the technical assistance of Miss Ann Robertson. This investigation was supported by a grant from the National Research Council of Canada.

References

1. Abell, M. R. and Beveridge, J. M. R. Can. J. Research, E, 27 : 316. 1949.
2. Abell, M. R. and Beveridge, J. M. R. Arch. Pathol. 52 : 428. 1951.
3. Binkley, F., Anslow, W. P., Jr., and DuVigneaud, V. J. Biol. Chem. 143 : 559. 1942.
4. Copher, G. H. and Dick, B. M. Arch. Surg. 17 : 408. 1928.
5. Daft, F. S., Sebrell, W. H., and Lillie, R. D. Proc. Soc. Exptl. Biol. Med. 50 : 1 1942.
6. Dam, H. and Granados, H. Acta Pharmacol. Toxicol. 7 : 167. 1951.
7. Glynn, L. E. and Himsworth, H. P. J. Pathol. Bacteriol. 56 : 297. 1944.
8. Gyorgy, P. and Goldblatt, H. J. Exptl. Med. 70 : 185. 1939.
9. Gyorgy, P. and Goldblatt, H. J. Exptl. Med. 89 : 245. 1949.
10. Gyorgy, P., Stokes, J., Jr., and Goldblatt, H. Trans. Assoc. Am. Physicians, 44 : 289. 1951.
11. Gyorgy, P., Stokes, J., Jr., Goldblatt, H., and Popper, H. J. Exptl. Med. 93 : 513. 1951.
12. Gyorgy, P., Stokes, J., Jr., Smith, H., and Goldblatt, H. Am. J. Med. Sci. 220 : 6 1950.
13. Himsworth, H. P. and Glynn, L. E. Clin. Sci. 5 : 93. 1944.
14. Himsworth, H. P. and Lindan, O. Nature, 163 : 30. 1949.
15. McLean, J. R. and Beveridge, J. M. R. Can. J. Med. Sci. 30 : 118. 1952.
16. McLean, J. R. and Beveridge, J. M. R. J. Nutrition, 47 : 41. 1952.
17. McLean, J. R. and Beveridge, J. M. R. J. Nutrition. In press.
18. Schwarz, K. Z. Physiol. Chem. (Hoppe-Seyler's), 281. 101. 1944.
19. Schwarz, K. Z. Physiol. Chem. (Hoppe-Seyler's), 281 : 109. 1944.
20. Schwarz, K. Federation Proc. 11 : 455. 1952.

PHYSIOLOGIC STUDIES OF THE GROUNDHOG (*MARMOTA MONAX*)[1]

By J. E. McBirnie, F. G. Pearson, G. A. Trusler, H. H. Karachi, and W. G. Bigelow

Abstract

An investigation of hibernation was carried out with groundhogs in order to study their unusual tolerance to very low body temperatures. The normal carbon dioxide content of the blood of groundhogs, even while they are in the nonhibernating phase, is higher than that of dogs and humans. In groundhogs, the transition from the nonhibernating to the hibernating phase involves a fall in body temperature to as low as 3° C. and appears to be associated with (1) an increase in the oxygen content and the carbon dioxide content of whole blood, (2) an elevation of the serum magnesium and serum potassium, (3) an increase in hematocrit values, and (4) a decrease in the blood sugar level. Direct microscopic examination of the peripheral circulation *in vivo* showed a free-flowing stream with no intravascular agglutination of red blood cells or vascular stasis in groundhogs, dogs, and rabbits at a body temperature of 20° C. or in groundhogs during hibernation at temperatures as low as 5° C.

Introduction

In the course of previous work (3–7), hypothermia has been induced in dogs by various methods. Physiological and biochemical studies have been carried out together with an investigation of an electrical pacemaker for cardiac standstill and a method for radio-frequency rewarming.

It became evident that despite the application of this knowledge, and with improved techniques of cooling and rewarming, induction of general hypothermia in dogs and monkeys below 20° C. was not yet a safe procedure. It was felt that a more fundamental approach to the problem was necessary and in view of the ability of certain hibernating animals to withstand body temperatures as low as 3° C. for prolonged periods, it was decided to investigate certain aspects of hibernation. By thus increasing our knowledge of general hypothermia, we hoped not only to facilitate its application to intracardiac surgery, but to improve current methods of resuscitating individuals exposed to severe cold.

To this end, observations were made comparing the blood gases, certain blood chemistry, and the microscopic peripheral circulation of nonhibernating and hibernating groundhogs. Comparative observations were also made on the microscopic peripheral circulation of dogs and rabbits at normal and low body temperatures.

[1] *Manuscript received June 3, 1953.*

Contribution from the Department of Surgery, Banting Institute, University of Toronto, Toronto, Ont.

This work was financed by a grant from the Defence Research Board of Canada.

The Groundhog

The groundhog (*Marmota monax*) which we have studied is a brown furred herbivorous animal averaging 2.5 to 3 kilo. in weight. He lives in burrows which he digs in fields to a depth of 2 to 6 ft.

In the autumn, the groundhog undergoes an alteration of metabolism in which there is a profound lowering of body temperature to within a degree or so of environmental temperature (often only three degrees Centigrade above freezing) with a concomitant decrease in oxygen consumption, cardiac rate, and respiratory rate. This seasonal change is influenced by environmental temperature, availability of food, and certain other factors such as the presence or absence of light or noise. In the torpid state, the animal can live for several months without the ingestion of food or water (1).

The groundhogs used in this study were obtained from southern Ontario by trapping or by digging the torpid animals from their burrows in the late fall or early spring. They were housed in a hut (12 ft. × 10 ft. × 10 ft.) erected on the roof of the Banting Institute. The temperature inside the hut was normally maintained between 5° C. and 10° C. by a thermostatically controlled electric heater. Owing to their strength and viciousness, the groundhogs were kept in individual cages constructed of steel and heavy galvanized wire mesh. Occasionally two animals were housed in the same cage without fighting. Excelsior was placed in each cage to serve as a nest. A mixture of canned creamed corn and corn meal, apples, cabbage, bread, and water comprised the diet.

Thick leather gauntlets were worn to protect the hands and the alert groundhog was handled by suspending the animal by its tail. No protection was necessary when handling the hibernating groundhog.

This report is most conveniently divided into two parts.

Part I. Observations to Compare the Blood Gases and Certain Blood Chemistry in the Nonhibernating and Hibernating Groundhog

Previous investigators (8, 11, 12, 14) have found that the carbon dioxide content in the blood of marmots was higher in hibernation than during the nonhibernating phase. They suggested that this might have a fundamental bearing on the mechanism of hibernation. Certainly on theoretical grounds, an increase of carbon dioxide tension increases the oxygen dissociation from oxyhemoglobin (Barcroft). This knowledge would be helpful in explaining how the tissues are adequately oxygenated at low body temperatures when hypothermia itself diminishes the oxygen dissociation of oxyhemoglobin (Brown and Hill).

Suomalainen (13) in studies on the hibernating hedgehog, concluded that insulin, blood sugar, and serum magnesium were intimately involved in the phenomenon of hibernation. In the hope that some basic facts might be

learned from a comparative study of the blood of hibernating and nonhibernating groundhogs, we obtained samples of blood from both active and torpid animals. On these samples the following determinations were made: oxygen and carbon dioxide content of arterial and venous blood, oxygen capacity of whole blood, serum magnesium, serum potassium, blood sugar, and hematocrit.

Methods

This work was carried out over a period of two years from 1950 to 1952.

During the first year, to obtain blood from groundhogs who were not in a state of hibernation, the animals were anesthetized with a minimal intraperitoneal injection of nembutal (approximately 1/3 grain per kilo.) and secured to the operating table. Using 5% cocaine anesthetic locally, the femoral vessels of one side were exposed and No. 5 French whistle tip ureteral catheters were passed up to the aorta and inferior vena cava. Blood samples totalling 15 cc. were drawn into heparinized syringes under oil.

Hibernating groundhogs of this series were removed from the cold hut into the laboratory, which had been previously cooled to between 10° C. and 15° C., and placed on the cooling blankets through which refrigerant at 1° C. was circulated in coils. They were completely unconscious and could not be roused. The operative procedure was accomplished, without anesthetic, in the manner described for the nonhibernating animals. With this technique, the torpid animal gained only 1° or 2° C. throughout the procedure, and could be operated on for one to one and a half hours without disturbance.

The magnesium, potassium, and hematocrit results shown are from specimens taken in the above manner. Blood gases and blood sugar estimations were also made but considered unreliable owing to the possibility of anesthetic effect in the nonhibernating animal and arousal changes in the hibernating animal.

Consequently the following year, the series was repeated with some changes. Nonhibernating groundhogs were anesthetized with a minimal intraperitoneal dose of 3% pentothal (1 cc. per kilogram) plus supplemental open ether. They were catheterized as before but now the catheters were tied in position and the wound tightly closed. Following suspension in a cloth and adhesive sling with the feet hanging free, the animal was allowed to recover completely from the anesthetic (see Fig. 1). The animal, on regaining consciousness, became accustomed to this suspended position and rested quietly, although completely awake, provided he was covered with a sheet and unable to observe the operator. While the animal was in this state, blood samples were taken, without disturbing the animal, a minimum of three hours after the administration of pentothal. In the four animals that had samples taken both 3 and 12 hr. following catheterization, the values were found to agree closely.

During the hibernating phase, only animals in deep winter sleep with a rectal temperature of between 4° and 10° C. were used. Within five minutes of removal of the torpid animal from the cold room, the aorta and inferior vena cava were catheterized and the blood samples withdrawn. Anesthesia

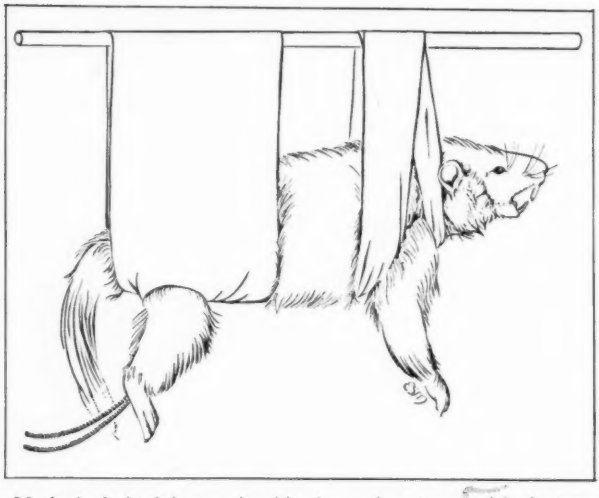


FIG. 1. Method of obtaining resting blood samples at normal body temperature.

was not required and the procedure caused but minimal disturbance, with an occasional stretching or yawning movement and an increase in respiratory rate from one to three or four per minute.

Samples were taken in heparinized syringes under oil and within one hour the blood gases and blood sugar determinations were made.

The carbon dioxide and oxygen content of blood was determined by the manometric method of Van Slyke and Neill. The Schoeffer-Hartmann method as modified by Harding was used for the determination of blood sugar. The serum potassium estimations were obtained after the method of Stohl and Bennett as modified by Consolazio and Talbott. Gomori's modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter was used to measure serum content of magnesium.

Observations

The blood gas estimations are summarized in Table I. They reveal an increase in the blood carbon dioxide content during hibernation compared to the content in the nonhibernating state. This is particularly noticeable in venous blood with an increased arteriovenous difference Although the oxygen content and oxygen saturation are similarly elevated, the oxygen capacity of the blood remains unchanged during hibernation.

The results of the blood chemistry estimations are summarized in Tables II and III. The groundhog's serum magnesium is elevated in the hibernating state. The content shows a marked rise as the animal enters hibernation and immediately declines in the posthibernation phase. The serum potassium levels are higher in the hibernating animals.

TABLE I

BLOOD GASES IN NONHIBERNATING AND HIBERNATING GROUNDHOGS

Animal No.	Date	CO_2, volumes %			O_2, volumes %			O_2, comb. cap. vol. % art.	O_2, sat. vol. % art.
		Art.	Ven.	Diff.	Art.	Ven.	Diff.		
Hibernating									
79	19.1.52	79.5	90.6	11.1	19.6	14.0	5.6	20.0	98
80	19.1.52	78.0	86.0	8.0	19.4	15.5	3.9	20.0	97
89	19.1.52	79.0	87.5	8.5	19.5	16.0	3.5	21.0	92
81	25.1.52	80.0	86.0	6.0	19.0	17.0	2.0	20.5	93
86	26.1.52	92.0	98.0	6.0	19.1	16.5	2.6	21.5	90
71	26.1.52	79.5	88.0	8.5	21.0	18.0	3.0	21.5	98
53	2.2.52	62.5	73.2	10.7	19.2	18.0	1.2	21.0	91
98	2.2.52	62.8	77.0	14.2	21.0	19.0	2.0	21.0	100
Average		76.6	85.8	9.2	19.7	16.7	3.0	20.8	95
Nonhibernating									
115	2.6.52	66.0	69.0	3.0	17.0	14.0	3.0	21.0	81
111	3.6.52	72.0	73.0	1.0	17.0	15.0	2.0	20.0	85
108	4.6.52	70.0	74.0	4.0					
116	4.6.52	65.0	70.0	5.0	17.0	16.0	1.0	19.0	89
116	4.6.52	68.0	71.0	3.0	17.0	16.0	1.0	19.4	87
103	6.6.52	63.0	68.0	5.0	18.0	15.0	3.0	19.5	92
118	16.6.52	76.0	80.0	4.0	19.0	15.6	3.4	21.0	90
118	16.6.52	74.0	77.0	3.0	18.6	16.0	2.6	21.0	88
117	17.6.52	72.0	78.0	6.0	17.7	16.5	1.2	20.0	85
117	17.6.52	70.0	78.0	8.0	18.0	17.0	1.0	20.0	90
118	18.6.52	76.0	83.0	7.0	18.5	15.0	3.5	21.0	88
118	18.6.52	75.0	80.0	5.0	18.0	15.0	3.0	20.5	87
Average		70.6	75.0	4.4	17.8	15.5	2.3	20.2	87

TABLE II

SERUM MAGNESIUM, SERUM POTASSIUM, AND HEMATOCRIT LEVELS IN GROUNDHOGS

Prehibernating phase, December, 1950		Hibernating phase, January, 1951				Posthibernating phase, April, 1951			
Groundhog, No.	Mg, mgm. %	Groundhog, No.	Mg, mgm. %	K, mgm. %	Hematocrit	Groundhog, No.	Mg, mgm. %	K, mgm. %	Hematocrit
2	2.6	12	4.4	17.6	49	27	3	8.2	35
5	2.3	11	4.7	17.4	43	29	3.4	9.4	36
3	3.1	1	4.0	21.2	48	30	3.8	8.0	36
4	3.3	10	4.8	14.5	49	31	2.9	10.0	25
5	2.3	9	4.3	22.0	56	33	2.2	11.7	27
2	2.5	7	4.5	13.7	48	35	3.8	11.3	29.5
Average	2.7	Average	4.4	17.7	48.8	Average	3.5	9.7	31.4

TABLE III

BLOOD SUGAR LEVELS IN GROUNDHOGS

Prehibernating phase			Hibernating phase			Posthibernating phase		
Animal	Month	Blood sugar, mgm. %	Animal	Month	Blood sugar, mgm. %	Animal	Month	Blood sugar, mgm. %
60	Sept./51	95	79	Jan./52	82	115	June/52	75
61	"	125	80	"	36	111	"	69
64	"	120	89	"	140	108	"	110
63	"	143	81	"	69	116 (1)	"	95
65	"	139	86	"	94	116 (2)	"	96
66	"	116	71	"	52	103	"	84
55	"	116	53	Feb./52	80	118 (1)	"	120
67	Oct./51	128	98	"	65	118 (2)	"	122
68	"	95				117 (1)	"	86
69	"	121				117 (2)	"	86
70	"	135						
Average		121	Average		66.8	Average		94.3
Range 95 – 135			Range 36 – 140			Range 69 – 122		

Blood sugars, although widely ranged, show a marked fall during hibernation. The values in September and October, just before hibernation are higher than those of June, just following hibernation and the rut.

Our findings indicate a rise in the hematocrit in the hibernating groundhog.

Discussion

In 1894, Dubois (8) noted that the blood carbon dioxide content of the European marmot was higher during hibernation. Rasmussen (11) in 1915 confirmed this observation, and also showed that the oxygen content of the blood underwent a similar elevation. More recently Svihla and Bowman (14) found this change in dormant ground squirrels. But for a lesser elevation of the oxygen content of blood during hibernation, our results in groundhogs agree closely with those of the latter authors. They also noted an increase in the red cell count and hematocrit, with a lowering of the blood volume during hibernation and suggested that the elevated blood gas figures were due to a hemoconcentration as a result of reduction in blood serum. Although we did not do blood volume studies, our hematocrit levels are consistent with this view. We are unable to explain however, the apparent contradiction of the increased hematocrit values without a corresponding rise in arterial oxygen capacity.

One striking observation in both nonhibernating and hibernating groundhogs is the high carbon dioxide content of the blood as compared with that in the dog or the human whose values range between 50 and 60 volumes per cent. This has been previously noted by other authors (8, 11, 14), not only in the groundhog, but also in the European marmot and the ground squirrel. As we have seen, this is greatest during hibernation and may well be a factor in counteracting the shift to the left of the oxygen dissociation curve of the blood *in vitro* at low temperatures.

This whole picture, with an increased concentration of circulating red blood cells and an elevated blood carbon dioxide and blood oxygen content, would seem to indicate an excellent adjustment of the hibernating animal towards preventing tissue hypoxia while allowing a substantial reduction of cardiac and respiratory action at low body temperatures.

The increased serum magnesium levels are consistent with Suomalainen's findings in torpid hedgehogs (13). Instead of the decreased serum potassium values which he describes, we found a rise during hibernation.

The blood sugar results are in close agreement with those of Suomalainen, who noted a pronounced hypoglycemia during hibernation.

Part II. A Comparative Microscopic Study of the Peripheral Circulation of the Groundhog and Certain Other Animals at Low Body Temperatures

It seldom has been possible to reduce the body temperature of dogs or monkeys below 16° C. without inducing ventricular fibrillation or cardiac standstill. The cardiac function of the groundhog appears to be little affected

by cold of a temperature of 3° C. It was our hypothesis that the former type of animal might possess a greater tendency to intravascular agglutination of erythrocytes during hypothermia with resultant blockage of peripheral circulation and hypoxia of tissues. If this were so, it might help to explain our inability to cool nonhibernating animals below 16° C.

The phenomenon of intravascular agglutination has been described elsewhere (2, 10). It is essentially a reaction which occurs in the blood following trauma and in most diseased states and is characterized chiefly by an agglutination of red blood cells. With an advanced degree of intravascular agglutination and particularly in the presence of vasospasm, these clumps of red blood cells have been seen to behave as emboli and to occlude finer units of the vascular bed throughout the body. In healthy animals and humans, the peripheral circulation flows freely and evenly in all vessels. The red and white cells remain discrete and pass through the capillary bed with ease.

The purpose of this study was to test our hypothesis by examining the peripheral circulation of groundhogs, dogs, and rabbits both at normal and at reduced body temperatures.

Methods

The animal was given a minimal dose of intravenous or intraperitoneal nembutal and cooled to approximately 20° C. in a water and ice bath. Endotracheal intubation and the administration of oxygen were made available as required. The omentum or mesentery was chosen to be studied. The sclera of the dog and the nictitating membrane of the rabbit were also used. These tissues were transilluminated by light conducted through a fused quartz rod previously described (2). Ringer's solution, at the same temperature as that of the animal being examined, flowed constantly over and under the tissue. A binocular, biobjective microscope was used to view the circulation.

Three groundhogs at lower body temperatures were examined using a similar technique—two of these animals were hibernating at 5° C. and 13° C. respectively (these required no anesthetic) and one was cooled artificially to 9° C.

The mesentery of one groundhog was examined at 35° C.

Results and Observations

The results of the experiments are tabulated in Table IV. These results show that in both the hibernating type (groundhog) and nonhibernating type animals (dog and rabbit) examined at 20° C., the blood flow, although occasionally slow, was consistently smooth and even with no evidence of intravascular agglutination or stasis.

Further, at the much reduced body temperatures attainable in the groundhog, i.e. 5°, 9°, and 13° C., the groundhog shows no tendency to intravascular agglutination. This observation obtained whether the animal was hypothermic from natural hibernation as in groundhogs No. 4 and No. 15, or whether hypothermia was artificially induced as in groundhog No. 2. This is

TABLE IV

TABULATION OF MICROSCOPIC OBSERVATIONS ON THE PERIPHERAL CIRCULATION OF HYPOTHERMIC GROUNDHOGS, DOGS, AND RABBITS. (OBSERVATIONS JANUARY TO APRIL, 1951.)

Animal	Body temperature, °C.	Intravascular agglutination
Hibernating groundhog No. 4	5	None
Hibernating groundhog No. 16	13	None
Groundhog No. 10	18	None
Groundhog No. 36	20	None
Groundhog No. 2	35	None
Dog No. 1	20.8	None
Dog No. 2	20.5	None
Rabbit No. 1	22	None
Rabbit No. 2	20	None

of interest in connection with the work of various authors who have found a prolonged clotting time and decreased prothrombin level in ground squirrels (15, 16) and an increase in mast cells in hedgehogs (9) during hibernation.

Summary and Conclusions

Part I

1. Blood gas and certain blood chemical estimations were made in alert nonhibernating groundhogs and compared with estimations made during deep hibernation.
2. The management of the animals, the technique for obtaining the blood samples, and the methods of analysis are described.
3. The normal carbon dioxide content of the blood of groundhogs while alert and nonhibernating is higher than the carbon dioxide content in dogs and humans.
4. The transition from the nonhibernating to the hibernating phase appears to be associated with: (1) an increase in the oxygen content and the carbon dioxide content of whole blood, (2) an elevation of the serum magnesium and serum potassium, (3) an increase in hematocrit values, and (4) a decrease in the blood sugar level.

Part II

1. The following hypothesis has been advanced to explain the inability of adult animals that do not hibernate (such as the dog) to tolerate body temperatures much below 16° C. Intravascular agglutination and vascular stasis induced by cold may produce sufficient peripheral vascular occlusion to cause death.

2. Intravascular agglutination of red blood cells and vascular stasis was not observed directly in the peripheral circulation of groundhogs, dogs, and rabbits at a body temperature of 20° C.
3. The peripheral circulation of groundhogs during hibernation at temperatures as low as 5° C. is free-flowing and shows no intravascular agglutination or stasis.
4. This phenomenon of intravascular agglutination did not provide an explanation for the great difference in cold tolerance between hibernating and nonhibernating type animals.

Acknowledgment

The authors would like to thank Prof. James Dauphinee and Dr. A. C. Gornall of the Department of Pathological Chemistry for their cooperation and Clarence Downs for valuable technical advice.

References

1. BENEDICT, F. G. and LEE, R. C. Carnegie Inst. Wash. Pub. 497. 1938.
2. BIGELOW, W. G., HEIMBECKER, R. O., and HARRISON, R. C. Arch. Surg. 59 : 667. 1949.
3. BIGELOW, W. G., HOPPS, J. A., and CALLAGHAN, J. C. Can. J. Med. Sci. 30 : 185. 1952.
4. BIGELOW, W. G., LINDSAY, W. K., and GREENWOOD, W. F. Ann. Surg. 132 : 849. 1950.
5. BIGELOW, W. G., LINDSAY, W. K., HARRISON, R. C., GORDON, R. A., and GREENWOOD, W. F. Am. J. Physiol. 160 : 125. 1950.
6. BIGELOW, W. G., MCBIRNIE, J. E., and KARACHI, H. H. Defence Research Board of Canada, Rept. No. DR 51. 1952.
7. CALLAGHAN, J. C. and BIGELOW, W. G. Ann. Surg. 134 : 8. 1951.
8. DUBOIS, R. Physiologie comparée de la marmotte. Paris. 246. 1896.
9. HARMA, R. and SOUMALAINEN, P. Acta Physiol. Scand. 24 : 90. 1951.
10. HEIMBECKER, R. O. and BIGELOW, W. G. Surgery, 28 : 461. 1950.
11. RASMUSSEN, A. T. Am. J. Physiol. 41 : 162. 1916.
12. STORMONT, R. T., FOSTER, M. A., and PFEIFFER, C. Proc. Soc. Exptl. Biol. Med. 42 : 56. 1939.
13. SOUMALAINEN, P. Arch. Soc. zool-botan. fenn-Vanamo, 5 : 35. 1950.
14. SVIHLA, A. and BOWMAN, H. C. Am. J. Physiol. 171 : 478. 1952.
15. SVIHLA, A., BOWMAN, H. C., and RITENAUR, R. Science, 114 : 298. 1951.
16. SVIHLA, A., BOWMAN, H. C., and RITENAUR, R. Science, 115 : 306. 1952.

THE INCIDENCE OF *E. COLI* GROUP 0111 IN SPORADIC INFANTILE GASTROENTERITIS AND THE SENSITIVITY TO ANTIBIOTICS OF THE STRAINS ISOLATED[1]

By Norman A. Hinton, J. E. Nelles, and G. B. Reed

Abstract

E. coli group 0111 was isolated in large numbers from the stools of 24 of 47 cases of infantile gastroenteritis. The *in vitro* sensitivity of 20 of these strains to seven antibacterial agents was tested and polymyxin B and aureomycin found to be the most effective.

Introduction

Since the observations of Bray (1) in 1945, epidemiological data have accumulated which support the concept of the etiological role of certain serotypes (0111; 055; 026)* of *E. coli* in epidemic and sporadic infantile gastroenteritis (7, 8, 11, 15, 21, 22, 24, 27, 29, 30, 31). These strains have been found to be present, often almost in pure culture, in the gastrointestinal tracts of many infants with diarrhea whereas the extensive series of Dupont (3) has shown that they are only rarely isolated from the stools of normal children.

Experimental feeding of these strains to infants and adults has resulted in gastrointestinal upset of varying severity although the ingestion of 'normal' strains is well tolerated (6, 9, 15, 16). Further, Neter and his associates have developed a sensitive hemagglutination test for the demonstration of antibodies to these *E. coli* serotypes (13, 14) and have shown that adult volunteers develop an increased, specific hemagglutination titer following feeding experiments (19).

Results which diverge from the main stream of evidence have, however, been recorded by Stevenson (28), Payne (20), and Cathie (2) and most authors are cautious in expressing the view that these serotypes bear a direct etiological relationship to infantile gastroenteritis although a recent editorial in the Lancet (4) states that the evidence at hand shows that the 0111 type of *E. coli* may cause both epidemic and sporadic infantile gastroenteritis.

Considering the marked variation in the occurrence of specific serotypes in different areas it was thought to be pertinent to determine the incidence of *E. coli* group 0111 in sporadic gastroenteritis in this area and the antibiotic sensitivity of the strains so isolated.

Material and Methods

The present study was carried out in the Pediatric Department of the Kingston General Hospital and the Department of Bacteriology of Queen's University during the period from Sept. 1, 1952, to May 1, 1953. In this

[1] *Manuscript received July 3, 1953.*

Contribution from the Department of Bacteriology, Queen's University, Kingston, Ontario, and the Department of Pediatrics, Kingston General Hospital.

* *These three strains have been so classified by Kauffman on the basis of their somatic antigens.*

interval 47 unselected, sporadic cases of gastroenteritis were studied along with a control group of 50 infants of like age admitted to the wards at the same time with some unrelated illness.

Rectal swabs were taken during the acute stage of the diarrhea, an ordinary sterile throat swab being introduced through an infant sized proctoscope and a sample obtained high up in the rectum. Twenty typical individual coliform colonies were selected from MacConkey rectal swab plates and tested for slide agglutination with *E. coli* 0111 serum prepared in rabbits and diluted 1 : 4 with normal saline. Two colonies from each positive stool were checked by a standard tube agglutination technique. In no case did a colony which gave a positive slide agglutination fail to agglutinate to the control titer.

The biochemical activity of 20 of the strains isolated was determined, incubation being carried out at 37° C. for a period of seven days and the cultures in sugar media being examined daily.

The sensitivity of these 20 strains to the antibacterial agents sulphadiazine, penicillin, streptomycin, aureomycin, chloromycetin, terramycin, and polymyxin B was determined by a broth dilution method. Five milliliter volumes of nutrient broth were distributed to a series of 6 in. by 5/8 in. culture tubes. Twice the desired maximum concentration of antibiotic was added to the first tube and serial dilution was carried out through the subsequent tubes. This procedure was followed in duplicate and all tubes inoculated with one drop of a 1 : 10 dilution of a 24 hr. broth culture of the test organism. The cultures were examined after 24 hr. incubation at 37° C. and the least concentration of the drug in which no visible growth occurred was taken as the end point of sensitivity.

In the clinical assessment of the severity of the various cases particular attention was paid to the frequency and nature of the stools, the duration of diarrhea and vomiting, the degree of dehydration, the presence of acidosis, and the presence or absence of parenteral infection. Cases were thus classified on this basis as mild, moderate, or severe.

The cases studied were admitted to the hospital by several different clinicians so that the choice of antibiotic varied with the individual. Basically all infants were taken off oral feedings for 24 hr. or longer and rehydrated as necessary with intravenous or interstitial fluids. Several of the severe cases were given blood transfusions.

TABLE I

CASES AND CONTROLS FROM WHICH E. *coli* GROUP 0111 WAS ISOLATED

Two month periods	Cases of gastroenteritis	Controls
1	2 of 6	0 of 10
2	7 of 13	0 of 10
3	8 of 15	2 of 15
4	7 of 13	2 of 15
Total period	24 of 47	4 of 50

TABLE IIA

CASES OF INFANTILE GASTROENTERITIS WITH *E. coli* GROUP 0111 *positive* STOOLS

Case number	Colonies positive/ 20 tested	Age in months	Duration of diarrhea	Severity of case	Previous diarrhea	Antibiotic therapy
32	20/20	3	14 days	Severe	Yes	Sulphonamide
45	20/20	1	21 days	Severe	No	Sulphonamide
46	20/20	1	40 days	Severe (died)	No	Sulphadiazine Terramycin
55	20/20	3	21 days	Severe	No	Sulphadiazine Streptomycin
62	20/20	2	28 days	Severe	No	Chloromycetin
64	20/20	1	25 days	Severe (died)	No	Terramycin
87	20/20	2	21 days	Severe	Yes	Sulphonamide
17	8/20	1	14 days	Severe	No	Streptomycin Terramycin
5	20/20	2	28 days	Moderate	No	Sulphonamide
26	20/20	10	10 days	Moderate	No	Sulphadiazine Terramycin
43	20/20	2	14 days	Moderate	No	Terramycin
57	20/20	3	10 days	Moderate	Yes	Sulphonamide
78	20/20	9	14 days	Moderate	Yes	Terramycin
79	20/20	4	16 days	Moderate	No	Terramycin
25	10/20	24	10 days	Moderate	Yes	Sulphonamide
13	10/20	5	6 days	Moderate	Yes	Sulphonamide
15	6/20	18	8 days	Moderate	No	Sulphonamide
21	20/20	2	7 days	Mild	No	Nil
22	20/20	1	6 days	Mild	Yes	Sulphonamide
59	20/20	2	10 days	Mild	No	Terramycin
49	20/20	3	21 days	Mild	Yes	Terramycin
23	3/20	14	8 days	Mild	No	Sulphonamide
53	4/20	4	7 days	Mild	No	Terramycin
36	20/20	No data				

TABLE IIB

CASES OF INFANTILE GASTROENTERITIS WITH *E. coli* GROUP 0111 *negative* STOOLS

Case number	Colonies positive/ 20 tested	Age in months	Duration of diarrhea	Severity of case	Previous diarrhea	Antibiotic therapy
29	0/20	6	18 days	Moderate	No	Sulphonamide Terramycin
86	0/20	12	7 days	Moderate	No	Sulphonamide Terramycin
11	0/20	1	8 days	Moderate	No	Nil
27	0/20	6	10 days	Moderate	No	Terramycin
50	0/20	7	11 days	Mild	No	Sulphonamide Terramycin
34	0/20	1	10 days	Mild	No	Sulphonamide Terramycin
33	0/20	3	10 days	Mild	No	Sulphonamide
16	0/20	3	7 days	Mild	No	Sulphonamide
28	0/20	8	7 days	Mild	No	Sulphonamide Terramycin
91	0/20	4	5 days	Mild	No	Terramycin
14	0/20	4	6 days	Mild	No	Terramycin
31	0/20	3	8 days	Mild	No	Sulphonamide
9	0/20	2	15 days	Mild	No	Sulphonamide
7	0/20	24	5 days	Mild	No	Sulphonamide
77	0/20	3	9 days	Mild	No	Sulphonamide
Eight further cases—data not available						

Results

Of the 47 cases 24 were positive, an incidence of approximately 50% (Table I). There were only four positive stools in the 50 nondiarrheal infants tested. Of the 24 cases of diarrhea which were positive, in 18 the test organism was found to be the predominant aerobic organism in the stool whereas in only one control stool was this found to be the case.

A consideration of Table II indicates that the disease was in general more severe and prolonged in those cases from which *E. coli* group 0111 was recovered. There appeared to be no relationship between the recovery of this organism and the incidence of parenteral infection. Eight of the positive cases had a history of previous diarrhea whereas none of the negative cases had such a history.

Biochemical Characteristics

All of the 20 strains of type 0111 tested demonstrated the conventional reactions of the *E. coli* group. The only differences were noted in the fermentation of sucrose and salicin. Two strains fermented both carbohydrates, the remainder did not ferment salicin. Eight strains fermented sucrose rapidly (within 24 hr.), nine strains fermented sucrose slowly (after five days), and three strains did not ferment sucrose. Thus three fermentative types were

TABLE III

ANTIBIOTIC SENSITIVITY OF 20 STRAINS OF *E. coli* GROUP 0111 ISOLATED FROM CASES OF INFANTILE DIARRHEA

Strain	Strepto-mycin	Aureo-mycin	Chloro-mycetin	Terramycin	Polymyxin	Sulpha-diazine
433	3.2	6.4	12.5	3.2	1.25	25.0
25	6.4	6.4	6.4	6.4	0.13	25.0
43	12.5	6.4	12.5	6.4	0.25	12.5
46A	>1000	6.4	25.0	6.4	0.32	25.0
46B	>1000	6.4	12.5	12.5	1.25	25.0
23	100	6.4	6.4	6.4	0.13	12.5
21	>1000	3.2	25.0	6.4	0.64	12.5
36	>1000	3.2	25.0	6.4	0.13	12.5
22	>1000	3.2	25.0	6.4	0.13	12.5
39	>1000	12.5	25.0	12.5	0.25	50.0
15	250	12.5	25.0	6.4	0.64	12.5
45	>1000	3.2	25.0	12.5	0.13	25.0
26	>1000	3.2	50.0	12.5	0.13	25.0
48	>1000	6.4	50.0	6.4	0.25	25.0
55	>1000	6.4	25.0	6.4	0.32	25.0
32	>1000	6.4	25.0	6.4	0.25	25.0
87	>1000	6.4	25.0	12.5	0.25	25.0
38	>1000	12.5	25.0	12.5	0.25	50.0
49	>1000	12.5	25.0	12.5	0.06	50.0
53	>1000	25.0	25.0	25.5	0.13	50.0

All strains grew well in 1000 units/ml. of penicillin. The numbers refer to the least concentration of the drug which completely inhibited growth after 24 hr. incubation. The concentration is measured in micrograms per ml. with the exception of sulphadiazine which is in milligrams %.

found among this serologically homogeneous group of organisms. Further biochemical studies revealed that 15 of the 20 strains tested belonged to the fermentative type *E. coli communius*.

Antibiotic Sensitivity

The sensitivity of 20 strains of *E. coli* group 0111 is recorded in detail in Table III. The extreme sensitivity of all strains to polymyxin B was striking. Aureomycin and terramycin appeared to be the most effective of the commonly used antibiotics although the strains tested were sensitive to all of the broad spectrum antibiotics. The sensitivity of these organisms to sulphadiazine was not remarkable and they were on the whole quite resistant to penicillin and streptomycin.

Discussion

The fact that in 18 of 47 cases of infantile gastroenteritis, the intestinal flora was almost completely replaced by a specific serotype of *E. coli* not commonly found in normal children would appear to be a significant observation. However, as Shanks (24), has remarked, although one can demonstrate serospecific strains of *E. coli* in unusual numbers in some cases of infantile diarrhea it does not necessarily mean that they play a causative role in the disease. The association of *E. coli* 0111 with the more severe cases is interesting but may only indicate that these strains are an aggravating factor. The history of diarrhea noted in eight of the positive cases may indicate that those children who harbor these serotypes may be more susceptible to repeated attacks of diarrhea regardless of the definitive etiology of the illness.

The *in vitro* sensitivity tests reported here confirm in general the findings expressed in the papers of Ferguson (5) and Smith (26). Although the test group is small the results are in agreement with Ferguson's contention that salicin nonfermenting strains are largely resistant to streptomycin. The variability of sensitivity of the different strains to the same antibiotic, and the variation in the sensitivity of any single strain to different antibiotics is such that, as Keating (10) has observed, the preliminary testing of each strain is necessary for rational therapy when attempting treatment of any infection due to the *Enterobacteriacae*.

In general, aureomycin and terramycin would appear to be the most effective of the common antibacterial agents for clinical use.

Acknowledgments

The strain of *E. coli* 0111 used in the preparation of rabbit immune serum was obtained through the courtesy of Dr. I. A. B. Cathie at the Great Ormond Street Hospital for Sick Children, London, England.

One of the authors (J. E. N.) wishes to thank the Banting Institute for financial assistance during the course of this work.

References

1. BRAY, J. J. Pathol. Bacteriol. 57 : 239. 1945.
2. CATHIE, I. A. B. and MACFARLANE, J. C. W. Brit. Med. J. 2 : 1002. 1951.
3. DUPONT, A. Brit. Med. J. 2 : 352. 1951.
4. EDITORIAL. Lancet, 2 : 323. 1952.
5. FERGUSON, W. W., JENNINGS, J. C., and GOTTSHALL, R. Y. Am. J. Hyg. 53 : 237. 1951.
6. FERGUSON, W. W. and JUNE, R. C. Am. J. Hyg. 55 : 155. 1952.
7. GILES, C. and SANGSTER, G. J. Hyg. 46 : 1. 1948.
8. GILES, C., SANGSTER, G., and SMITH, J. Arch. Disease Childhood, 24 : 45. 1949.
9. JUNE, R. C., FERGUSON, W. W., and WORFEL, M. T. Am. J. Hyg. 57 : 2. 1953.
10. KEATING, S. V. Med. J. Australia, 238 : 709. 1951.
11. KIRBY, A. C., HALL, E. C., and COAKLEY, W. Lancet, 2 : 201. 1950.
12. MAGNUSSON, J. H., LAURELL, G., FRISELL, E., and WARNER, B. Brit. Med. J. 1 : 1398. 1950.
13. NETER, E., BERTRAM, L. F., and ARBESMAN, C. E. Proc. Soc. Exptl. Biol. Med. 79 : 255. 1952.
14. NETER, E., BERTRAM, L. F., ZAC, D. A., MURDOCK, M. R., and ARBESMAN, C. E. J. Exptl. Med. 96 : 1. 1952.
15. NETER, E. and SHUMWAY, C. N. Proc Soc. Exptl. Biol. Med. 75 : 504. 1950.
16. NETER, E. and WEBB, C. R. Exptl. Med. Surg. 9 : 385. 1951.
17. NETER, E., WEBB, C. R., SHUMWAY, C. N., and MURDOCK, M. R. Am. J. Public Health, 41 : 1490. 1951.
18. NETER, E., ZAC, D. A., ZALEWSKI, N. J., and BERTRAM, L. F. Proc. Soc. Exptl. Biol. Med. 80 : 607. 1952.
19. NETER, E., ZALEWSKI, N. J., and FERGUSON, W. W. Proc. Soc. Exptl. Biol. Med. 82 : 215. 1953.
20. PAYNE, A. M. M. and COOK, G. T. Brit. Med. J. 2 : 192. 1950.
21. ROGERS, K. B. Proc. Roy. Soc. Med. 44 : 519. 1951.
22. ROGERS, K. B., KOEGLER, S. J., and GERRARD, J. Brit. Med. J. 2 : 1501. 1949.
23. SCOTT, R. B., BROWN, G. P., and KESSLER, A. D. Am. J. Diseases Children, 83 : 192. 1952.
24. SHANKS, R. A. and STUDZINSKI, L. P. Brit. Med. J. 2 : 119. 1952.
25. SMELLIE, J. M. Proc. Roy. Soc. Med. 43 : 766. 1950.
26. SMITH, J. and GALLOWAY, W. H. Arch. Disease Childhood, 28 : 30. 1953.
27. SMITH, J., GALLOWAY, W. H., and SPEIRS, A. L. J. Hyg. 48 : 472. 1950.
28. STEVENSON, J. S. Brit. Med. J. 2 : 195. 1950.
29. STEVENSON, J. S. Brit. Med. J. 2 : 123. 1952.
30. TAYLOR, J. Proc. Roy. Soc. Med. 44 : 516. 1951.
31. TAYLOR, J., POWELL, B. W., and WRIGHT, J. Brit. Med. J. 2 : 117. 1949.

CANADIAN JOURNAL OF MEDICAL SCIENCES

Notes to Contributors

GENERAL: Manuscripts should be typewritten, double spaced, and the original and one extra copy submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. If authors require changes to be made after the type is set, they will be charged for changes that are considered to be excessive. **All pages, whether text, figures, or tables, should be numbered.**

ABSTRACT: **An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.**

ILLUSTRATIONS:

(i) *Line Drawings:* All lines should be of sufficient thickness to reproduce well. Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper **ruled in blue only**; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in **green, yellow, or red should not be used** unless it is desired to have all the co-ordinate lines show. Lettering and numerals should be neatly done in India ink preferably with a stencil (**do not use typewriting**) and be of such size that they will be legible and not less than one millimeter in height when reproduced in a cut three inches wide. All experimental points should be carefully drawn with instruments. Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. **The original drawings and one set of small but clear photographic copies are to be submitted.**

(ii) *Photographs:* Prints should be made on glossy paper, with strong contrasts; they should be trimmed to remove all extraneous material so that essential features only are shown. Photographs should be submitted **in duplicate**; if they are to be reproduced in groups, one set should be so arranged and mounted on cardboard with rubber cement; the duplicate set should be unmounted.

(iii) *General:* **The author's name, title of paper, and figure number should be written in the lower left hand corner (outside the illustration proper) of the sheets on which the illustrations appear.** Captions should not be written on the illustrations, but typed together at the end of the manuscript. All figures (including each figure of the plates) should be numbered consecutively from 1 up (arabic numerals). **Each figure should be referred to in the text.** If authors desire to alter a cut, they will be charged for the new cut.

TABLES: Each table should be typed on a separate sheet. Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum. **Each table should be referred to in the text.**

REFERENCES: **These should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper.** The form of literature citation should be that used in this Journal. **Titles of papers in the** references cited should **not** be given. Initial page numbers only are required. **All citations should be checked with the original articles.** Each citation should be referred to in the text by means of the key number.

REPRINTS: A total of 50 reprints of each paper, without covers, are supplied free to the authors. Additional reprints will be supplied according to a prescribed schedule of charges. On request, covers can be supplied at cost.

Approximate charges for reprints may be calculated from the number of printed pages, obtained by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ in. × 11 in.) and making allowance for space occupied by line drawings and halftones (not inserts). The cost per page is tabulated on the back of the reprint request form sent with the galley.

Contents

Page

Slide Culture of Tubercle Bacilli. I. Media and Methods—*Roger W. Reed* - - 367

On Rat Serum Amylase. I. Studies in the Normal and the Alloxan Diabetic Animal—*Jules Tuba and Stuart Wiberg* - - - - - - - 377

A Method for the Determination of the Effects of Drugs on the Pain Threshold of Human Subjects—*G. A. Deneau, R. A. Waud, and C. W. Goudey* - - 387

The Combination of Heparin and Bovine Serum Albumin—*D. W. Clarke and F. C. Monkhouse* - - - - - - - - - - - - 394

The Effect of Age, Sex, and Previous Pregnancy on Oxygen Uptake of Mammary Tissue of Mice of the C_{57}BL, A, and C_3H Strains—*Jules Tuba, Herbert E. Rawlinson, Lorna G. Shaw, M. Shirley Fraser, and Irmgaard Jeske* - - 400

Statistical Methods in Medical Research. II. Sample Sizes Required in Experiments Involving All-or-none Responses — *Donald Mainland and Marion I. Sutcliffe* - - - - - - - - - - - 406

The Prevention of Liver Necrosis by Alpha-tocopherol, Methionine, and Cysteine by Oral and Parenteral Routes—*J. R. McLean and J. M. R. Beveridge* - - - - - - - - - - - - - 417

Physiologic Studies of the Groundhog (*Marmota monax*)—*J. E. McBirnie, F. G. Pearson, G. A. Trusler, H. H. Karachi, and W. G. Bigelow* - - - 421

The Incidence of *E. coli* Group 0111 in Sporadic Infantile Gastroenteritis and the Sensitivity to Antibiotics of the Strains Isolated—*Norman A. Hinton, J. E. Nelles, and G. B. Reed* - - - - - - - - 431